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(54) **Recombinant polypeptides and peptides, nucleic acids coding for the same and use of these polypeptides and peptides in the diagnostic of tuberculosis.**

(57) The invention relates to recombinant polypeptides and peptides and particularly to the polypeptide containing in its polypeptidic chain the following amino acid sequence:
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 4a and fig. 4b.

The polypeptides and peptides of the invention can be used for the diagnostic of tuberculosis, and can also be part of the active principle in the preparation of vaccine against tuberculosis.

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RECOMBINANT POLYPEPTIDES AND PEPTIDES, NUCLEIC ACIDS CODING FOR THE SAME AND USE OF THESE POLYPEPTIDES AND PEPTIDES IN THE DIAGNOSTIC OF TUBERCULOSIS

The invention relates to recombinant polypeptides and peptides, which can be used for the diagnosis of tuberculosis. The invention also relates to a process for preparing the above-said polypeptides and peptides, which are in a state of biological purity such that they can be used as part of the active principle in the preparation of vaccines against tuberculosis.

5 It also relates to nucleic acids coding for said polypeptides and peptides.

Furthermore, the invention relates to the in vitro diagnostic methods and kits using the above-said polypeptides and peptides and to the vaccines containing the above-said polypeptides and peptides as active principle against tuberculosis.

By "recombinant polypeptides or peptides" it is to be understood that it relates to any molecule having
10 a polypeptidic chain liable to be produced by genetic engineering, through transcription and translation, of a corresponding DNA sequence under the control of appropriate regulation elements within an efficient cellular host. Consequently, the expression "recombinant polypeptides" such as is used herein does not exclude the possibility for the polypeptides to comprise other groups, such as glycosylated groups.

The term "recombinant" indeed involves the fact that the polypeptide has been produced by genetic
15 engineering, particularly because it results from the expression in a cellular host of the corresponding nucleic acid sequences which have previously been introduced into the expression vector used in said host.

Nevertheless, it must be understood that this expression does not exclude the possibility for the polypeptide to be produced by a different process, for instance by classical chemical synthesis according to methods used in the protein synthesis or by proteolytic cleavage of larger molecules.

20 The expression "biologically pure" or "biological purity" means on the one hand a grade of purity such that the recombinant polypeptide can be used for the production of vaccinating compositions and on the other hand the absence of contaminants, more particularly of natural contaminants.

Tuberculosis remains a major disease in developing countries. The situation is dramatic in some countries, particularly where high incidence of tuberculosis among AIDS patients represents a new source
25 of dissemination of the disease.

Tuberculosis is a chronic infectious disease in which cell-mediated immune mechanisms play an essential role both for protection against and control of the disease.

Despite BCG vaccination, and some effective drugs, tuberculosis remains a major global problem. Skin testing with tuberculin PPD (protein-purified derivative) largely used for screening of the disease is poorly
30 specific, due to cross reactivity with other pathogenic or environmental saprophytic mycobacteria.

Moreover, tuberculin PPD when used in serological tests (ELISA) does not allow to discriminate between patients who have been vaccinated by BCG, or those who have been primo-infected, from those who are developing evolutive tuberculosis and for whom an early and rapid diagnosis would be necessary.

A protein with a molecular weight of 32-kDa has been purified (9) from zinc deficient Mycobacterium
35 bovis BCG culture filtrate (8). This 32-kDa protein of M. bovis BCG has been purified from Sauton zinc deficient culture filtrate of M. bovis BCG using successively hydrophobic chromatography on Phenyl-Sephadex, ion exchange on DEAE-Sephacel and molecular sieving on Sephadex G-100. The final preparation has been found to be homogeneous as based on several analyses. This P₃₂ protein is a constituent of BCG cells grown in normal conditions. It represents about 3% of the soluble fraction of a
40 cellular extract, and appears as the major protein released in normal Sauton culture filtrate. This protein has been found to have a molecular weight of 32 000 by SDS-polyacrylamide gel electrophoresis and by molecular sieving.

The NH₂-terminal amino acid sequence of the 32-kDa protein of M. bovis BCG (Phe-Ser-Arg-Pro-Gly-Leu) is identical to that reported for the MPB 59 protein purified from M. bovis BCG substrain Tokyo (34).

45 Purified P₃₂ of M. bovis BCG has been tested by various cross immunoelectrophoresis techniques, and has been shown to belong to the antigen 85 complex in the reference system for BCG antigens. It has been more precisely identified as antigen 85A in the cross reference system for BCG antigens (7).

Increased levels of immunoglobulin G antibodies towards the 32-kDa protein of M. bovis BCG could be detected in 70% of tuberculous patients (30).

50 Furthermore, the 32-kDa protein of M. bovis BCG induces specific lymphoproliferation and interferon-(IFN- γ) production in peripheral blood leucocytes from patients with active tuberculosis (12) and PPD-positive healthy subjects. Recent findings indicate that the amount of 32-kDa protein of M. bovis BCG-induced IFN- γ in BCG-sensitized mouse spleen cells is under probable H-2 control (13). Finally, the high affinity of mycobacteria for fibronectin is related to proteins of the BCG 85 antigen complex (1).

Matsuo et al. (17) recently cloned the gene encoding the antigen α , a major protein secreted by BCG (substrain Tokyo) and highly homologous to MPB 59 antigen in its NH₂-terminal amino acid sequence, and even identical for its first 6 amino acids : Phe-Ser-Arg-Pro-Gly-Leu.

This gene was cloned by using a nucleotide probe homologous to the N-terminal amino acid sequence of antigen α , purified from *M. tuberculosis* as described in Tasaka, H. et al., 1983. "Purification and antigenic specificity of alpha protein (Yoneda and Fukui) from *Mycobacterium tuberculosis* and *Mycobacterium intracellulare*. Hiroshima J. Med. Sci. 32, 1-8.

The presence of antigens of around 30-32-kDa, named antigen 85 complex, has been revealed from electrophoretic patterns of proteins originating from culture media of mycobacteria, such as *Mycobacterium tuberculosis*. By immunoblotting techniques, it has been shown that these antigens cross-react with rabbit sera raised against the 32-kDa protein of BCG (8).

A recent study reported on the preferential humoral response to a 30-kDa and 31-kDa antigen in lepromatous leprosy patients, and to a 32-kDa antigen in tuberculoid leprosy patients (24).

It has also been found that fibronectin (FN)-binding antigens are prominent components of short-term culture supernatants of *Mycobacterium tuberculosis*. In 3-day-old supernatants, a 30-kilodalton (kDa) protein was identified as the major (FN)-binding molecule. In 21-day-old supernatants, FN was bound to a double protein band of around 30 to 32-kDa, as well as to a group of antigens of larger molecular mass (57 to 60 kDa)(1).

In other experiments, recombinant plasmids containing DNA from *Mycobacterium tuberculosis* were transformed into *Escherichia coli*, and three colonies were selected by their reactivity with polyclonal antisera to *M. tuberculosis*. Each recombinant produced 35- and 53-kilodalton proteins (35K and 53K proteins, respectively) ("Expression of Proteins of *Mycobacterium tuberculosis* in *Escherichia coli* and Potential of Recombinant Genes and Proteins for Development of Diagnostic Reagents", Mitchell L Cohen et al., *Journal of Clinical Microbiology*, July 1987, p.1176-1180).

Concerning the various results known to date, the physico-chemical characteristics of the antigen P₃₂ of *Mycobacterium tuberculosis* are not precise and, furthermore, insufficient to enable its unambiguous identifiability, as well as the characterization of its structural and functional elements.

Moreover, the pathogenicity and the potentially infectious property of *M. tuberculosis* has hampered research enabling to identify, purify and characterize the constituents as well as the secretion products of this bacteria.

An aspect of the invention is to provide recombinant polypeptides which can be used as purified antigens for the detection and control of tuberculosis.

Another aspect of the invention is to provide nucleic acids coding for the peptidic chains of biologically pure recombinant polypeptides which enable their preparation on a large scale.

Another aspect of the invention is to provide antigens which can be used in serological tests as an *in vitro* rapid diagnostic of tuberculosis.

Another aspect of the invention is to provide a rapid *in vitro* diagnostic means for tuberculosis, enabling it to discriminate between patients suffering from an *evolutive* tuberculosis from those who have been vaccinated against BCG or who have been *primo*-infected.

Another aspect of the invention is to provide nucleic probes which can be used as *in vitro* diagnostic reagent for tuberculosis, as well as *in vitro* diagnostic reagent for identifying *M. tuberculosis* from other strains of mycobacteria.

The recombinant polypeptides of the invention contain in their polypeptidic chain one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 3a and fig. 3b, or

- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :
- 5 the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or
- react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,
- and/or react with the amino acid sequence extending from the extremity constituted by amino acid at
- 10 position (1), to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

On figures 3a and 3b :

- X represents G or GG,
- Y represents C or CC,
- Z represents C or G,
- 15 - W represents C or G and is different from Z,
- X represents C or CG,
- L represents G or CG,
- a₁-b₁ represents ALA-ARG or GLY-ALA-ALA,
- a₂ represents arg or gly,
- 20 - a₃-b₃-c₃-d₃-e₃-f₃- represents his-trp-val-pro-arg-pro or ala-leu-gly-ala,
- a₄ represents pro or pro-asn-thr,
- a₅ represents pro or ala-pro.

The recombinant polypeptides of the invention contain in their polypeptidic chain one at least of the following amino acid sequences:

- 25 - the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity
- 30 constituted by amino acid at position (55) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 4a and fig. 4b, or
- 35 - the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity
- 40 constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :
- the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or
- 45 react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,
- and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

The recombinant polypeptides of the invention contain in their polypeptidic chain one at least of the following amino acid sequences:

- 50 - the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (-1) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 5, or
- 55 - the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 5, or

- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 5, or
- 5 - the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (295) represented on fig. 5,

and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :
 10 the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or

react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,

15 and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (295) represented on fig. 5.

Advantageous polypeptides of the invention are characterized by the fact that they react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, hereafter designated by "P₃₂ protein of BCG".

20 Advantageous polypeptides of the invention are characterized by the fact that they selectively react with human sera from tuberculous patients and particularly patients developing an evolutive tuberculosis at an early stage.

Hereafter is given, in a non limitative way a process for preparing rabbit polyclonal antiserum raised against the P₃₂ protein of BCG and a test for giving evidence of the reaction between the polypeptides of
 25 the invention and said rabbit polyclonal antiserum raised against the P₃₂ protein of BCG.

1) process for preparing rabbit polyclonal antiserum raised against the P₃₂ protein of BCG:

30 Purified P₃₂ protein of BCG from culture filtrate is used.

a) Purification of protein P₃₂ of BCG

35 P₃₂ protein can be purified as follows :

The bacterial strains used are M. bovis BCG substrains 1173P2 (Pasteur Institute, Paris) and GL2 (Pasteur Institute, Brussels).

The culture of bacteria is obtained as follows :

40 Mycobacterium bovis BCG is grown as a pellicle on Sauton medium, at 37.5° C for 14 days. As the medium is prepared with distilled water, zinc sulfate is added to the final concentration of 5 µM (normal Sauton medium) (De Bruyn J., Weckx M., Beumer-Jochmans M.-P. Effect of zinc deficiency on Mycobacterium tuberculosis var. bovis (BCG). J. Gen. Microbiol. 1981; 124:353-7). When zinc deficient medium was needed, zinc sulfate is omitted.

The filtrates from zinc deficient cultures are obtained as follows :

45 The culture medium is clarified by decantation. The remaining bacteria are removed by filtration through Millipak 100 filter unit (Millipore Corp., Bedford, Mass.). When used for purification, the filtrate is adjusted to 20 mM in phosphate, 450 mM in NaCl, 1 mM in EDTA, and the pH is brought to 7.3 with 5 M HCl before sterile filtration.

50 The protein analysis is carried out by polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done on 13% (w/v) acrylamide-containing gels as described by Laemmli UK. (Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5). The gels are stained with Coomassie Brilliant Blue R-250 and for quantitative analysis, scanned at 595 nm with a DU8 Beckman spectrophotometer. For control of purity the gel is revealed with silver stain (Biorad Laboratories, Richmond, Calif.).

55 The purification step of P₃₂ is carried out as follows:

Except for hydrophobic chromatography on Phenyl-Sepharose, all buffers contain Tween 80 (0.005% final concentration). The pH is adjusted to 7.3 before sterilization. All purification steps are carried out at +4° C. Elutions are followed by recording the absorbance at 280 nm. The fractions containing proteins are

analysed by SDS-PAGE.

(i) The treated filtrate from a 4 liters zinc-deficient culture, usually containing 125 to 150 mg protein per liter, is applied to a column (5.0 by 5.0 cm) of Phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden), which is previously equilibrated with 20 mM phosphate buffer (PB) containing 0.45 M NaCl and 1 mM EDTA, at a flow rate of 800 ml per hour. The gel is then washed with one column volume of the same buffer to remove unfixed material and successively with 300 ml of 20 mM and 4 mM PB and 10% ethanol (v/v). The P_{32} appears in the fraction eluted with 10% ethanol.

(ii) After the phosphate concentration of this fraction has been brought to 4 mM, it is applied to a column (2.6 by 10 cm) of DEAE-Sephacel (Pharmacia Fine Chemicals), which is equilibrated with 4 mM PB. After washing with the equilibrating buffer the sample is eluted with 25 mM phosphate at a flow rate of 50 ml per hour. The eluate is concentrated in a 202 Amicon stirred cell equipped with a PM 10 membrane (Amicon Corp., Lexington, Mass.).

(iii) The concentrated material is submitted to 4 mg of P_{32} protein of BCG (soluble extract) or molecular sieving on a Sephadex G-100 (Pharmacia) column (2.6 by 45 cm) equilibrated with 50 mM PB, at a flow rate of 12 ml per hour. The fractions of the peak giving one band in SDS-PAGE are pooled. The purity of the final preparation obtained is controlled by SDS-PAGE followed by silverstaining and by molecular sieving on a Superose 12 (Pharmacia) column (12.0 by 30 cm) equilibrated with 50 mM PB containing 0.005% Tween 80 at a flow rate of 0.2 ml/min. in the Fast Protein Liquid Chromatography system (Pharmacia). Elution is followed by recording the absorbance at 280 nm and 214 nm.

b) Preparation of rabbit polyclonal antiserum raised against the P_{32} protein of BCG :

400 μ g of purified P_{32} protein of BCG per ml physiological saline are mixed with one volume of incomplete Freund's adjuvant. The material is homogenized and injected intradermally in 50 μ l doses delivered at 10 sites in the back of the rabbits, at 0, 4, 7 and 8 weeks (adjuvant is replaced by the diluent for the last injection). One week later, the rabbits are bled and the sera tested for antibody level before being distributed in aliquots and stored at -80° C;

2) test for giving evidence of the reaction between the polypeptides of the invention and said rabbit polyclonal antiserum raised against the P_{32} protein of BCG:

the test used was an ELISA test; the ELISA for antibody determination is based on the method of Engvall and Perlmann (Engvall, E., and P. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin C. Immunochemistry 8:871-874)

Immulon Microelisa plates (Dynatech, Kloten, Switzerland) are coated by adding to each well 1 μ g of one of the polypeptides of the invention in 100 μ l Tris hydrochloride buffer 50 mM (pH 8.2). After incubation for 2 h at 27° C in a moist chamber, the plates are kept overnight at 4° C. They are washed four times with 0.01 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 by using a Titertek microplate washer (Flow Laboratories, Brussels, Belgium). Blocking is done with 0.5% gelatin in 0.06 M carbonate buffer (pH 9.6) for 1 h. Wells are then washed as before, and 100 μ l of above mentioned serum diluted in phosphate-buffered saline containing 0.05% Tween 20 and 0.5% gelatin is added. According to the results obtained in preliminary experiments, the working dilutions are set at 1:200 for IgG, 1:20 for IgA and 1:80 for IgM determinations. Each dilution is run in duplicate. After 2 h of incubation and after the wells are washed, they are filled with 100 μ l of peroxidase-conjugated rabbit immunoglobulins directed against human IgG, IgA or IgM (Dakopatts, Copenhagen, Denmark), diluted 1:400, 1:400 and 1:1.200, respectively in phosphate-buffered saline containing 0.05% Tween 20 and 0.5% gelatin and incubated for 90 min. After the wash, the amount of peroxidase bound to the wells is quantified by using a freshly prepared solution of o-phenylenediamine (10 mg/100 ml) and hydrogen peroxide (8 μ l of 30% H_2O_2 per 100 ml) in 0.15 M citrate buffer (pH 5.0) as a substrate. The enzymatic reaction is stopped with 8 N H_2SO_4 after 15 min. of incubation. The optical density is read at 492 nm with a Titertek Multiskan photometer (Flow Laboratories).

Wells without sera are used as controls for the conjugates. Each experiment is done by including on each plate one negative and two positive reference sera with medium and low antibody levels to correct for plate-to-plate and day-to-day variations. The antibody concentrations are expressed as the optical density values obtained after correction of the readings according to the mean variations of the reference sera.

Hereafter is also given in a non limitative way, a test for giving evidence of the fact that polypeptides of the invention are recognized selectively by human sera from tuberculous patients.

This test is an immunoblotting (Western blotting) analysis, in the case where the polypeptides of the invention are obtained by recombinant techniques. This test can also be used for polypeptides of the invention obtained by a different preparation process. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, polypeptides of the invention are blotted onto nitrocellulose membranes (Hybond C. (Amersham)) as described by Towbin et al. (29). The expression of polypeptides of the invention fused to β -galactosidase in *E. coli* Y1089, is visualized by the binding of a polyclonal rabbit anti-32-kDa BCG protein serum (1:1,000) or by using a monoclonal anti- β -galactosidase antibody (Promega). The secondary antibody (alkaline phosphatase anti-rabbit immunoglobulin G and anti-mouse alkaline phosphatase immunoglobulin G conjugates, respectively) is diluted as recommended by the supplier (Promega).

In order to identify selective recognition of polypeptides of the invention and of fusion proteins of the invention by human tuberculous sera, nitrocellulose sheets are incubated overnight with these sera (1:50) (after blocking aspecific protein-binding sites). The human tuberculous sera are selected for their reactivity (high or low) against the purified 32-kDa antigen of BCG tested in a dot blot assay as described in document (31) of the bibliography hereafter. Reactive areas on the nitrocellulose sheets are revealed by incubation with peroxidase conjugated goat anti-human immunoglobulin G antibody (Dakopatts, Copenhagen, Denmark) (1:200) for 4h, and after repeated washings, color reaction is developed by adding peroxidase substrate (α -chloronaphtol) (Bio-Rad Laboratories, Richmond, Calif.) in the presence of peroxidase and hydrogen peroxide.

It goes without saying that the free reactive functions which are present in some of the amino acids, which are part of the constitution of the polypeptides of the invention, particularly the free carboxyl groups which are carried by the groups Glu or by the C-terminal amino acid on the one hand and/or the free NH_2 groups carried by the N-terminal amino acid or by amino acid inside the peptidic chain, for instance Lys, on the other hand, can be modified in so far as this modification does not alter the above mentioned properties of the polypeptide.

The molecules which are thus modified are naturally part of the invention. The above mentioned carboxyl groups can be acylated or esterified.

Other modifications are also part of the invention. Particularly, the amine or ester functions or both of terminal amino acids can be themselves involved in the bond with other amino acids. For instance, the N-terminal amino acid can be linked to a sequence comprising from 1 to several amino acids corresponding to a part of the C-terminal region of another peptide.

Furthermore, any peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids of the polypeptides according to the invention are part of the invention in so far as this modification does not alter the above mentioned properties of said polypeptides.

The polypeptides according to the invention can be glycosylated or not, particularly in some of their glycosylation sites of the type Asn-X-Ser or Asn-X-Thr, X representing any amino acid.

Advantageous recombinant polypeptides of the invention contain in their polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b.

Advantageous recombinant polypeptides of the invention contain in their polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b.

- the one extending from the extremity constituted by amino acid at position (-43) to the extremity constituted by amino acid at position (-1) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (-1) represented on fig. 5.

5 In eukaryotic cells, these polypeptides can be used as signal peptides, the role of which is to initiate the translocation of a protein from its site of synthesis, but which is excised during translocation.

Other advantageous peptides of the invention consist in one of the following amino acid sequence:

- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 3a and fig. 3b, or
- 10 - the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 3a and fig. 3b, or
- 15 - the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 3a and fig. 3b, or
- 20 - the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

Other advantageous peptides of the invention consist in one of the following amino acid sequence:

- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 4a and fig. 4b, or
- 25 - the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 4a and fig. 4b, or
- 30 - the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 4a and fig. 4b, or
- 35 - the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

Other advantageous peptides of the invention consist in one of the following amino acid sequence:

- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 5, or
- 40 - the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 5, or
- 45 - the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 5, or
- 50 - the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (295) represented on fig. 5.

It is to be noted that the above mentioned polypeptides are derived from the expression products of a DNA derived from the nucleotide sequence coding for a protein of 32-kDa secreted by *Mycobacterium tuberculosis* as explained hereafter in the examples.

55 The invention also relates to the amino acid sequences constituted by the above mentioned polypeptides and a protein or an heterologous sequence with respect to said polypeptide, said protein or heterologous sequence comprising for instance from about 1 to about 1000 amino acids. These amino acid sequences will be called fusion proteins.

In an advantageous fusion protein of the invention, the heterologous protein is β -galactosidase.

Other advantageous fusion proteins of the invention are the ones containing an heterologous protein resulting from the expression of one of the following plasmids:

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pEX1

pEX2

pEX3

pUEX1

pmTNF MPH

pUEX2

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pUEX3

The invention also relates to any nucleotide sequence coding for a polypeptide of the invention.

The invention also relates to nucleic acids comprising nucleotide sequences which hybridize with the
15 nucleotide sequences coding for any of the above mentioned polypeptides under the following hybridization conditions:

- hybridization and wash medium: 3 X SSC, 20% formamide (1 X SSC is 0,15 M NaCl, 0,015 M sodium citrate, pH 7.0),
- hybridization temperature (HT) and wash temperature (WT) for the nucleic acids of the invention defined
20 by x-y: i.e. by the sequence extending from the extremity constituted by the nucleotide at position (x) to the extremity constituted by the nucleotide at position (y) represented on fig. 3a and fig. 3b.

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1 - 182	HT = WT 69 °C
1 - 194	HT = WT 69 °C
1 - 212	HT = WT 69 °C
1 - 218	HT = WT 69 °C
1 - 272	HT = WT 69 °C
1 - 359	HT = WT = 71 °C
1 - 1241	HT = WT = 73 °C
1 - 1358	HT = WT = 73 °C
183 - 359	HT = WT = 70 °C
183 - 1241	HT = WT = 73 °C
183 - 1358	HT = WT = 73 °C
195 - 359	HT = WT = 70 °C
195 - 1241	HT = WT = 73 °C
195 - 1358	HT = WT = 73 °C
213 - 359	HT = WT = 70 °C
213 - 1241	HT = WT = 73 °C
213 - 1358	HT = WT = 73 °C
219 - 359	HT = WT = 71 °C
219 - 1241	HT = WT = 73 °C
219 - 1358	HT = WT = 73 °C
234 - 359	HT = WT = 71 °C
234 - 1241	HT = WT = 74 °C
234 - 1358	HT = WT = 73 °C
273 - 359	HT = WT = 71 °C
273 - 1241	HT = WT = 74 °C
273 - 1358	HT = WT = 73 °C
360 - 1241	HT = WT = 73 °C
360 - 1358	HT = WT = 73 °C
1242 - 1358	HT = WT = 62 °C

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The above mentioned temperatures are to be considered as approximately ± 5 °C.

The invention also relates to nucleic acids comprising nucleotide sequences which are complementary to the nucleotide sequences coding for any of the above mentioned polypeptides.

It is to be noted that in the above defined nucleic acids, as well as in the hereafter defined nucleic

acids, the nucleotide sequences which are brought into play are such that T can be replaced by U.

A group of preferred nucleic acids of the invention comprises one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 3a and fig. 3b,

or above said nucleotide sequences wherein T is replaced by U,

- or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.

A group of preferred nucleic acids of the invention comprises one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 4a and fig. 4b,

or above said nucleotide sequences wherein T is replaced by U,

- or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.

A group of preferred nucleic acids of the invention comprises one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1104) to the extremity constituted by nucleotide at position (1299), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 5,

or above said nucleotide sequences wherein T is replaced by U,

- or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.

Other preferred nucleic acids of the invention comprise one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b.

Other preferred nucleic acids of the invention comprise one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity

constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b.

Another preferred group of nucleic acids of the invention comprises the following nucleotide sequences:

- 5 - the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b.

Another preferred group of nucleic acids of the invention comprises the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b.

According to another advantageous embodiment, nucleic acids of the invention comprises one of the following sequences:

- [illegible]

According to another advantageous embodiment, nucleic acids of the invention comprises one of the following sequences:

- 55 - the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted

by nucleotide at position (194) represented in fig. 4a and fig. 4b,

- [illegible]

- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1299) represented in fig. 5.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (129) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1104) to the extremity constituted by nucleotide at position (1299) represented in fig. 5.

The invention also relates to any recombinant nucleic acids containing at least a nucleic acid of the invention inserted in an heterologous nucleic acid.

The invention relates more particularly to recombinant nucleic acid such as defined, in which the nucleotide sequence of the invention is preceded by a promoter (particularly an inducible promoter) under the control of which the transcription of said sequence is liable to be processed and possibly followed by a sequence coding for transcription termination signals.

The invention also relates to the recombinant nucleic acids in which the nucleic acid sequences coding for the polypeptide of the invention and possibly the signal peptide, are recombined with control elements which are heterologous with respect to the ones to which they are normally associated within the bacteria gene and, more particularly, the regulation elements adapted to control their expression in the cellular host which has been chosen for their production.

The invention also relates to recombinant vectors, particularly for cloning and/or expression, comprising a vector sequence, notably of the type plasmid, cosmid or phage, and a recombinant nucleic acid of the invention, in one of the non essential sites for its replication.

Appropriate vectors for expression of the recombinant antigen are the following one:

pEX1 pmTNF MPH
pEX2 pIGRI
pEX3
pUEX1
pUEX2
pUEX3

The pEX1, pEX2 and pEX3 vectors are commercially available and can be obtained from Boehringer Mannheim.

The pUEX1, pUEX2 and pUEX3 vectors are also commercially available and can be obtained from Amersham.

According to an advantageous embodiment of the invention, the recombinant vector contains, in one of its non essential sites for its replication, necessary elements to promote the expression of polypeptides according to the invention in a cellular host and possibly a promoter recognized by the polymerase of the cellular host, particularly an inducible promoter and possibly a signal sequence and/or an anchor sequence.

According to another additional embodiment of the invention, the recombinant vector contains the elements enabling the expression by *E. coli* of a nucleic acid according to the invention inserted in the vector, and particularly the elements enabling the expression of the gene or part thereof of β -galactosidase.

The invention also relates to a cellular host which is transformed by a recombinant vector according to the invention, and comprising the regulation elements enabling the expression of the nucleotide sequence coding for the polypeptide according to the invention in this host.

The invention also relates to a cellular host chosen from among bacteria such as *E. coli*, transformed by a vector as above defined, and defined hereafter in the examples, or chosen from among eukaryotic organism, such as CHO cells, insect cells, Sf9 cells [*Spodoptera frugiperda*] infected by the virus Ac NPV (Autographa californica nuclear polyhydrosis virus) containing suitable vectors such as pAc 373 pYM1 or pVC3, BmN [*Bombyx mori*] infected by the virus BmNPV containing suitable vectors such as pBE520 or p89B310.

The invention relates to an expression product of a nucleic acid expressed by a transformed cellular host according to the invention.

The invention also relates to nucleotidic probes, hybridizing with anyone of the nucleic acids or with their complementary sequences, and particularly the probes chosen among the following nucleotidic sequences gathered in Table 1, and represented in fig. 9.

TABLE 1

Probes A(i), A(ii), A(iii), A(iv) and A(v)

A(i) CAGCTTGTGACAGGGTTCGTGGC
A(ii) GGTTCGTGGCGCCGTCACG
A(iii) CGTCGCGCGCCTAGTGTCGG
A(iv) CGGCGCCGTCGGTGGCACGGCGA
A (v) CGTCGGCGCGGCCCTAGTGTCGG

Probe B
TCGCGCGCCCTGTACCTG

Probe C
GCGCTGACGCTGGCGATCTATC

Probe D
CCGCTGTTGAACGTCGGGAAG

Probe E
AAGCGGTCGGATCTGGGTGGCAAC

Probes F(i), F(ii), F(iii) and F(iv)

F (i) ACGGCACTGGGTGCCACGCCCAAC
F(ii) ACGCCCAACACGGGGCCCGCCGCA
F (iii) ACGGGCACTGGGTGCCACGCCCAAC
F(iv) ACGCCCAACACGGGGCCCGCCGCCCCA
or their complementary nucleotidic sequences.

The hybridization conditions can be the following ones:

- hybridization and wash medium: 3 X SSC, 20% formamide (1 X SSC is 0,15 M NaCl, 0.015 M sodium citrate, pH 7.0),
- hybridization temperature (HT) and wash temperature (WT):

(WT) °C:	HT and WT (°C)
A(i)	50
A(ii)	50
A(iii)	52
A(iv)	60
A(v)	52
B	48
C	50
D	45
E	52
F(i)	55
F(ii)	59
F(iii)	55
F(iv)	59

These probes might enable to differentiate M. tuberculosis from other bacterial strains and in particular from the following mycobacteria species:

- Mycobacterium marinum, Mycobacterium scrofulaceum, Mycobacterium gordonae, Mycobacterium szulgai, Mycobacterium intracellulare, Mycobacterium xenopi, Mycobacterium gastri, Mycobacterium nonchromogenicum, Mycobacterium terrae and Mycobacterium triviale, and more particularly from M. bovis, Mycobacterium kansasii, Mycobacterium avium, Mycobacterium phlei and Mycobacterium fortuitum.

The invention also relates to DNA or RNA primers which can be used for the synthesis of nucleotidic sequences according to the invention by PCR (polymerase chain reaction technique), such as described in US Patents n° 4,683,202 and n° 4,683,195 and European Patent n° 200362.

The invention also relates to any DNA or RNA primer constituted by about 15 to about 25 nucleotides of a nucleotide sequence coding for a polypeptide according to the invention.

The invention also relates to any DNA or RNA primer constituted by about 15 to about 25 nucleotides liable to hybridize with a nucleotide sequence coding for a polypeptide according to the invention.

The invention also relates to any DNA or RNA primer constituted by about 15 to about 25 nucleotides complementary to a nucleotide sequence coding for a polypeptide according to the invention.

The sequences which can be used as primers are given in Table 2 hereafter (sequences P1 to P6 or their complement) and illustrated in fig. 9 :

TABLE 2

P1	GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCC
P2	ATCAACACCCCGGCGTTCGAGTGGTAC
P2 compl.	GTACCACTCGAACGCCGGGGTGTTGAT
P3	TGCCAGACTTACAAGTGGGA
P3 compl.	TCCCACTTGTAAGTCTGGCA
P4	TCCTGACCAGCGAGCTGCCG
P4 compl.	CGGCAGCTCGCTGGTCAGGA
P5	CCTGATCGGCCTGGCGATGGGTGACGC
P5 compl.	GCGTCACCCATCGCCAGGCCGATCAGG
P6 compl.	GCGCCCCAGTACTCCCAGCTGTGCGT
compl. = complement	

The sequences can be combined in twelve different primer-sets (given in Table 3) which allow enzymatical amplification by the polymerase chain reaction (PCR) technique of any of the nucleotide sequences of the invention, and more particularly the one extending from the extremity constituted by nucleotide at position 1 to the extremity constituted by nucleotide at position 1358, as well as the nucleotide sequence of antigen α of BCG (17).

The detection of the PCR amplified product can be achieved by a hybridization reaction with an oligonucleotide sequence of at least 10 nucleotides which is located between PCR primers which have been used to amplify the DNA.

The PCR products of the nucleotide sequences of the invention can be distinguished from the α -antigen gene of BCG or part thereof by hybridization techniques (dot-spot, Southern blotting, etc.) with the probes indicated in Table 3. The sequences of these probes can be found in Table 1 hereabove.

TABLE 3

Primer set	Detection with probe
1. P1 and the complement of P2	B
2. P1 and the complement of P3	B
3. P1 and the complement of P4	B
4. P1 and the complement of P5	B or C
5. P1 and the complement of P6	B, C, D or E
6. P2 and the complement of P5	C
7. P2 and the complement of P6	C, D or E
8. P3 and the complement of P5	C
9. P3 and the complement of P6	C, D or E
10. P4 and the complement of P5	C
11. P4 and the complement of P6	C, D or E
12. P5 and the complement of P6	D or E

It is to be noted that enzymatic amplification can also be achieved with all oligonucleotides with sequences of about 15 consecutive bases of the primers given in Table 2. Primers with elongation at the 5'-end or with a small degree of mismatch may not considerably affect the outcome of the enzymatic amplification if the mismatches do not interfere with the base-pairing at the 3'-end of the primers.

Specific enzymatic amplification of the nucleotide sequences of the invention and not of the BCG gene can be achieved when the probes (given in Table 1) or their complements are used as amplification primers.

When the above mentioned probes of Table 1 are used as primers, the primer sets are constituted by any of the nucleotide sequences (A, B, C, D, E, F) of Table 1 in association with the complement of any other nucleotide sequence, chosen from A, B, C, D, E or F, it being understood that sequence A means any of the sequences A(i), A(ii), A(iii), A(iv), A(v) and sequence F, any of the sequences F(i), F(ii), F(iii) and F(iv).

Advantageous primer sets for enzymatic amplification of the nucleotide sequence of the invention can be one of the following primer sets given in Table 3bis hereafter:

TABLE 3BIS

	A(i)	
5	or A(ii)	
	or A(iii)	and the complement of B
	or A(iv)	
10	or A(v)	
	A(i)	
	or A(ii)	
	or A(iii)	and the complement of C
15	or A(iv)	
	or A(v)	
	B	and the complement of C
20	A(i)	
	or A(ii)	
	or A(iii)	and the complement of F
25	or A(iv)	
	or A(v)	

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	A(i)	
	or A(ii)	
5	or A(iii)	and the complement of D
	or A(iv)	
	or A(v)	
10	A(i)	
	or A(ii)	
	or A(iii)	and the complement of E
	or A(iv)	
15	or A(v)	
	B	and the complement of D
	B	and the complement of E
20	B	and the complement of F
	C	and the complement of D
	C	and the complement of E
25	C	and the complement of F
	D	and the complement of E
	D	and the complement of F
30	E	and the complement of F
	A(i), A(ii), A(iii), A(iv), A(v), B, C, D, E and F	
	having the nucleotide sequence indicated in Table 1.	

35 In the case of amplification of a nucleotide sequence of the invention with any of the above mentioned primer sets defined in Table 3bis hereabove, the detection of the amplified nucleotide sequence can be achieved by a hybridization reaction with an oligonucleotide sequence of at least 10 nucleotides, said sequence being located between the PCR primers which have been used to amplify the nucleotide sequence. An oligonucleotide sequence located between said two primers can be determined from figure 9
40 where the primers A, B, C, D, E and F are represented by the boxed sequences respectively named probe region A, probe region B, probe region C, probe region D, probe region E and probe region F.

The invention also relates to a kit for enzymatic amplification of a nucleotide sequence by PCR technique and detection of the amplified nucleotide sequence containing
45 - one of the PCR primer sets defined in Table 3 and one of the detection probes of the invention, advantageously the probes defined in Table 1,
or one of the PCR primer sets defined in Table 3bis, and a detection sequence consisting for instance in an oligonucleotide sequence of at least 10 nucleotides, said sequence being located (fig. 9) between the two PCR primers constituting the primer set which has been used for amplifying said nucleotide sequence.

The invention also relates to a process for preparing a polypeptide according to the invention
50 comprising the following steps:

- the culture in an appropriate medium of a cellular host which has previously been transformed by an appropriate vector containing a nucleic acid according to the invention,
- the recovery of the polypeptide produced by the above said transformed cellular host from the above said culture medium, and
- 55 - the purification of the polypeptide produced, eventually by means of immobilized metal ion affinity chromatography (IMAC).

The polypeptides of the invention can be prepared according to the classical techniques in the field of peptide synthesis.

The synthesis can be carried out in homogeneous solution or in solid phase.

For instance, the synthesis technique in homogeneous solution which can be used is the one described by Houbenweyl in the book titled "Methode der organischen chemie" (Method of organic chemistry) edited by E. Wunsh, vol. 15-I et II. THIEME, Stuttgart 1974.

5 The polypeptides of the invention can also be prepared according to the method described by R.D. MERRIFIELD in the article titled "Solid phase peptide synthesis" (J.P. Ham.Socks. , 45 , 2149-2154).

The invention also relates to a process for preparing the nucleic acids according to the invention.

A suitable method for chemically preparing the single-stranded nucleic acids (containing at most 100 nucleotides of the invention) comprises the following steps :

10 - DNA synthesis using the automatic β -cyanoethyl phosphoramidite method described in Bioorganic Chemistry 4; 274-325, 1986.

In the case of single-stranded DNA, the material which is obtained at the end of the DNA synthesis can be used as such.

A suitable method for chemically preparing the double-stranded nucleic acids (containing at most 100 bp of the invention) comprises the following steps:

15 - DNA synthesis of one sense oligonucleotide using the automatic β -cyanoethyl phosphoramidite method described in Bioorganic Chemistry 4; 274-325, 1986, and DNA synthesis of one anti-sense oligonucleotide using said above-mentioned automatic β -cyanoethyl phosphoramidite method,
 - combining the sense and anti-sense oligonucleotides by hybridization in order to form a DNA duplex,
 20 - cloning the DNA duplex obtained into a suitable plasmid vector and recovery of the DNA according to classical methods, such as restriction enzyme digestion and agarose gel electrophoresis.

A method for the chemical preparation of nucleic acids of length greater than 100 nucleotides - or bp, in the case of double-stranded nucleic acids -comprises the following steps :

25 - assembling of chemically synthesized oligonucleotides, provided at their ends with different restriction sites, the sequences of which are compatible with the succession of amino acids in the natural peptide, according to the principle described in Proc. Nat. Acad. Sci. USA 80; ,7461-7465, 1983,
 - cloning the DNA thereby obtained into a suitable plasmid vector and recovery of the desired nucleic acid according to classical methods, such as restriction enzyme digestion and agarose gel electrophoresis.

30 The invention also relates to antibodies themselves formed against the polypeptides according to the invention.

It goes without saying that this production is not limited to polyclonal antibodies.

It also relates to any monoclonal antibody produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or rat, immunized against the purified polypeptide of the invention on the one hand, and of cells of a myeloma cell line on the other hand.
 35 and to be selected by its ability to produce the monoclonal antibodies recognizing the polypeptide which has been initially used for the immunization of the animals.

The invention also relates to any antibody of the invention labeled by an appropriate label of the enzymatic, fluorescent or radioactive type.

40 The peptides which are advantageously used to produce antibodies, particularly monoclonal antibodies, are the following ones gathered in Table 4:

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TABLE 4a (see fig. 4a and 4b)

5	Amino acid position (NH ₂ -terminal)		Amino acid position (COOH-terminal)
	12	QVPSPSMGRDIKVQFQSGGA	31
10	36	LYLLDGLRAQDDFSGWDINT	55
	77	SFYSDWYQPACRKAGCQTYK	96
	101	LTSELPGWLQANRHVKPTGS	120
15	175	KASDMWGPKEPAWQRNDPL	194
	211	CGNGKPSDLGGNNLPAKFLE	230
	275	KPDLQRHWVPRPTGPPQGA	294

20

TABLE 4b (see fig. 5)

25	Amino acid position (NH ₂ -terminal)		Amino acid position (COOH-terminal)
30	77	SFYSDWYQPACGKAGCQTYK	96
	276	PDLQRALGATPNTGPAPQGA	295

35 The amino acid sequences are given in the 1-letter code.

40 Variations of the peptides listed in Table 4 are also possible depending on their intended use. For example, if the peptides are to be used to raise antisera, the peptides may be synthesized with an extra cysteine residue added. This extra cysteine residue is preferably added to the amino terminus and facilitates the coupling of the peptide to a carrier protein which is necessary to render the small peptide immunogenic. If the peptide is to be labeled for use in radioimmune assays, it may be advantageous to synthesize the protein with a tyrosine attached to either the amino or carboxyl terminus to facilitate iodination. These peptides possess therefore the primary sequence of the peptides listed in Table 4 but with additional amino acids which do not appear in the primary sequence of the protein and whose sole function is to confer the desired chemical properties to the peptides.

45 The invention also relates to a process for detecting *in vitro* antibodies related to tuberculosis in a human biological sample liable to contain them, this process comprising

- contacting the biological sample with a polypeptide or a peptide according to the invention under conditions enabling an *in vitro* immunological reaction between said polypeptide and the antibodies which are possibly present in the biological sample and

50 - the *in vitro* detection of the antigen/antibody complex which may be formed.

Preferably, the biological medium is constituted by a human serum.

The detection can be carried out according to any classical process.

By way of example a preferred method brings into play an immunoenzymatic process according to ELISA technique or immunofluorescent or radioimmunological (RIA) or the equivalent ones.

55 Thus the invention also relates to any polypeptide according to the invention labeled by an appropriate label of the enzymatic, fluorescent, radioactive... type.

Such a method for detecting *in vitro* antibodies related to tuberculosis comprises for instance the following steps:

- deposit of determined amounts of a polypeptidic composition according to the invention in the wells of a titration microplate,
- introduction into said wells of increasing dilutions of the serum to be diagnosed,
- incubation of the microplate,
- 5 - repeated rinsing of the microplate,
- introduction into the wells of the microplate of labeled antibodies against the blood immunoglobulins,
- the labeling of these antibodies being carried out by means of an enzyme which is selected from among the ones which are able to hydrolyze a substrate by modifying the absorption of the radiation of this latter at least at a given wave length,
- 10 - detection by comparing with a control standard of the amount of hydrolyzed substrate.

The invention also relates to a process for detecting and identifying in vitro antigens of M. tuberculosis in a human biological sample liable to contain them, this process comprising:

- contacting the biological sample with an appropriate antibody of the invention under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. tuberculosis which are
- 15 possibly present in the biological sample and the in vitro detection of the antigen/antibody complex which may be formed.

Preferably, the biological medium is constituted by sputum, pleural effusion liquid, broncho-alveolar washing liquid, urine, biopsy or autopsy material.

- 20 Appropriate antibodies are advantageously monoclonal antibodies directed against the peptides which have been mentioned in Table 4.

The invention also relates to an additional method for the in vitro diagnostic of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis comprising the following steps:

- the possible previous amplification of the amount of the nucleotide sequences according to the invention, liable to be contained in a biological sample taken from said patient by means of a DNA primer set as
- 25 above defined,
- contacting the above mentioned biological sample with a nucleotide probe of the invention, under conditions enabling the production of an hybridization complex formed between said probe and said nucleotide sequence,
- detecting the above said hybridization complex which has possibly been formed.

- 30 To carry out the in vitro diagnostic method for tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis as above defined, the following necessary or kit can be used, said necessary or kit comprising:

- a determined amount of a nucleotide probe of the invention,
- advantageously the appropriate medium for creating an hybridization reaction between the sequence to be
- 35 detected and the above mentioned probe,
- advantageously, reagents enabling the detection of the hybridization complex which has been formed between the nucleotide sequence and the probe during the hybridization reaction.

The invention also relates to an additional method for the in vitro diagnostic of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis comprising :

- 40 - contacting a biological sample taken from a patient with a polypeptide or a peptide of the invention, under conditions enabling an in vitro immunological reaction between said polypeptide or peptide and the antibodies which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which has possibly been formed.

- 45 To carry out the in vitro diagnostic method for tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis, the following necessary or kit can be used, said necessary or kit comprising:

- a polypeptide or a peptide according to the invention,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complex which has been produced by the immunological reaction, said reagents possibly having a label, or being liable to be recognized by a labeled reagent, more
- 50 particularly in the case where the above mentioned polypeptide or peptide is not labeled.

The invention also relates to an additional method for the in vitro diagnostic of tuberculosis in a patient liable to be infected by M. tuberculosis , comprising the following steps:

- contacting the biological sample with an appropriate antibody of the invention under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. tuberculosis which are
- 55 possibly present in the biological sample and - the in vitro detection of the antigen/antibody complex which may be formed.

Appropriate antibodies are advantageously monoclonal antibodies directed against the peptides which have been mentioned in Table 4.

To carry out the in vitro diagnostic method for tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis, the following necessary or kit can be used, said necessary or kit comprising:

- an antibody of the invention,
- reagents for making a medium appropriate for the immunological reaction to occur,
- 5 - reagents enabling to detect the antigen/antibody complexes which have been produced by the immunological reaction, said reagent possibly having a label or being liable to be recognized by a label reagent, more particularly in the case where the above mentioned antibody is not labeled.

An advantageous kit for the diagnostic in vitro of tuberculosis comprises:

- at least a suitable solid phase system, e.g. a microtiter-plate for deposition thereon of the biological sample to be diagnosed in vitro,
- 10 - a preparation containing one of the monoclonal antibodies of the invention,
- a specific detection system for said monoclonal antibody,
- appropriate buffer solutions for carrying out the immunological reaction between a test sample and said monoclonal antibody on the one hand, and the bonded monoclonal antibodies and the detection system on
- 15 the other hand.

The invention also relates to a kit, as described above, also containing a preparation of one of the polypeptides or peptides of the invention, said antigen of the invention being either a standard (for quantitative determination of the antigen of M. tuberculosis which is sought) or a competitor, with respect to the antigen which is sought, for the kit to be used in a competition dosage process.

- 20 The invention also relates to an immunogenic composition comprising a polypeptide or a peptide according to the invention, in association with a pharmaceutically acceptable vehicle.

The invention also relates to a vaccine composition comprising among other immunogenic principles anyone of the polypeptides or peptides of the invention or the expression product of the invention, possibly coupled to a natural protein or to a synthetic polypeptide having a sufficient molecular weight so that the conjugate is able to induce in vivo the production of antibodies neutralizing Mycobacterium tuberculosis, or induce in vivo a cellular immune response by activating M. tuberculosis antigen-responsive T cells.

The peptides of the invention which are advantageously used as immunogenic principle have one of the following sequences:

30 **TABLE 4a (see fig. 4a and 4b)**

35	Amino acid position (NH ₂ -terminal)		Amino acid position (COOH-terminal)
	12	QVPSPSMGRDIKVQFQSGGA	31
40	36	LYLLDGLRAQDDFSGWDINT	55
	77	SFYSDWYQPACRKAGCQTYK	96
	101	LTSELPGWLQANRHVKPTGS	120
	175	KASDMWGPKEPAWQRNDPL	194
45	211	CGNGKPSDLGGNNLPAKFLE	230
	275	KPDLQRHWVPRPTPGPPQGA	294

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TABLE 4b (see fig. 5)

5	Amino acid position (NH ₂ -terminal)		Amino acid position (COOH-terminal)
10	77	SFYSDWYQPACGKAGCQTYK	96
	276	PDLQRALGATPNTGPAPQGA	299

The amino acid sequences are given in the 1-letter code.

15 Other characteristics and advantages of the invention will appear in the following examples and the figures illustrating the invention.

Figures 1(A) and 1(B) correspond to the identification of six purified λ gt11 *M. tuberculosis* recombinant clones. Figure 1(A) corresponds to the EcoRI restriction analysis of clone 15, clone 16, clone 17, clone 19, clone 24 and EcoRI-HindIII digested lambda DNA-molecular weight marker lane (in kilobase pairs) (M) (Boehringer).

20 Figure 1(B) corresponds to the immunoblotting analysis of crude lysates of *E. coli* lysogenized with clone 15, clone 16, clone 17, clone 19, clone 23 and clone 24.

Arrow (\leftarrow) indicates fusion protein produced by recombinant λ gt11-M-tuberculosis clones. Expression and immunoblotting were as described above. Molecular weight (indicated in kDa) were estimated by comparison with molecular weight marker (High molecular weight-SDS calibration kit, Pharmacia).

25 Figure 2 corresponds to the restriction map of the DNA inserts in the λ gt11 *M. tuberculosis* recombinant clones 17 and 24 identified with polyclonal anti-32-kDa (BCG) antiserum as above defined and of clones By1, By2 and By5 selected by hybridization with a 120 bp EcoRI-Kpn I restriction fragment of clone 17.

30 DNA was isolated from λ gt11 phage stocks by using the Lambda Sorb Phage Immunoabsorbent, as described by the manufacturer (Promega). Restriction sites were located as described above. Some restriction sites were deduced from a computer analysis of the nucleotide sequence.

The short vertical bars



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represent linker derived EcoRI sites surrounding the DNA inserts of recombinant clones. The lower part represents a magnification of the DNA region containing the antigen of molecular weight of 32-kDa, that has been sequenced. Arrows indicate strategies and direction of dideoxy-sequencing. (\rightarrow) fragment subcloned in Bluescribe M13; ($\leftarrow\rightarrow$) fragment subcloned in mp10 and mp11 M13 vectors; ($\blacksquare\rightarrow$) sequence determined with the use of a synthetic oligonucleotide.

Figures 3a and 3b correspond to the nucleotide and amino acid sequences of the general formula of the antigens of the invention.

45 Figures 4a and 4b correspond to the nucleotide and amino acid sequences of one of the antigens of the invention.

Two groups of sequences resembling the *E. coli* consensus promoter sequences are boxed and the homology to the consensus is indicated by italic bold letters. Roman bold letters represent a putative Shine-Dalgarno motif.

50 The NH₂-terminal amino acid sequence of the mature protein which is underlined with a double line happens to be very homologous - 29/32 amino acids - with the one of MPB 59 antigen (34). Five additional ATG codons, upstream of the ATG at position 273 are shown (dotted underlined). Vertical arrows (!) indicate the presumed NH₂ end of clone 17 and clone 24. The option taken here arbitrarily represents the 59 amino acid signal peptide corresponding to ATG₁₈₃.

55 Figure 5 corresponds to the nucleotide and amino acid sequences of the antigen of 32-kDa of the invention.

The NH₂-terminal amino acid sequence of the mature protein which is underlined with a double line happens to be very homologous - 29/32 amino acids - with the one of MPB 59 antigen (34). Vertical arrows (!) indicate the presumed NH₂ end of clone 1,7 and clone 24.

Figure 6 is the hydropathy pattern of the antigen of the invention of a molecular weight of 32-kDa and of the antigen α of BCG (17).

Figure 7 represents the homology between the amino acid sequences of the antigen of 32-kDa of the invention and of antigen α of BCG (revised version).

5 Identical amino acids; (:) evolutionarily conserved replacement of an amino acid (.), and absence of homology () are indicated. Underlined sequence (=) represents the signal peptide, the option taken here arbitrarily representing the 43-amino acid signal peptide corresponding to ATG₉₁. Dashes in the sequences indicate breaks necessary for obtaining the optimal alignment.

10 Figure 8 illustrates the fact that the protein of 32-kDa of the invention is selectively recognized by human tuberculous sera.

Figure 8 represents the immunoblotting with human tuberculous sera, and anti- β -galactosidase monoclonal antibody. Lanes 1 to 6: *E. coli* lysate expressing fusion protein (140 kDa); lanes 7 to 12: unfused β -galactosidase (114 kDa). The DNA insert of clone 17 (2.7 kb) was subcloned into pUEX₂ and expression of fusion protein was induced as described by Bresson and Stanley (4). Lanes 1 and 7 were probed with the
15 anti- β -galactosidase monoclonal antibody; lanes 4, 5, 6 and 10, 11, 12 with 3 different human tuberculous sera highly responding towards purified protein of the invention of 32-kDa; lanes 2 and 3 and 8 and 9 were probed with 2 different low responding sera.

Figure 9 represents the nucleic acid sequence alignment of the 32-kDa protein gene of *M. tuberculosis* of the invention (upper line), corresponding to the sequence in fig. 5, of the gene of fig. 4a and 4b of the
20 invention (middle line), and of the gene for antigen α of BCG (lower line).

Dashes in the sequence indicate breaks necessary for obtaining optimal alignment of the nucleic acid sequence.

The primer regions for enzymatical amplification are boxed (P1 to P6).

25 The specific probe regions are boxed and respectively defined by probe region A, probe region B, probe region C, probe region D, probe region E and probe region F

It is to be noted that the numbering of nucleotides is different from the numbering of figures 3a and figure 3b, and of figure 5, because nucleotide at position 1 (on figure 9) corresponds to nucleotide 234 on Figure 3a, and corresponds to nucleotide 91 on figure 5.

30 Figure 10a corresponds to the restriction and genetic map of the pIGRI plasmid used in Example IV for the expression of the P₃₂ antigen of the invention in *E. coli*.

On this figure, underlined restriction sites are unique.

Figure 10b corresponds to the pIGRI nucleic acid sequence.

On this figure, the origin of nucleotide stretches used to construct plasmid pIGRI are specified hereafter.

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Position

5	3422-206 :	lambda PL containing EcoRI blunt-MboII blunt fragment of pPL(λ) (Pharmacia)
	207-384 :	synthetic DNA sequence
10	228-230 :	initiation codon ATG of first cistron
	234-305 :	DNA encoding amino acids 2 to 25 of mature mouse TNF
15	306-308 :	stop codon (TAA) first cistron
	311-312 :	initiation codon (ATG) second cistron
20	385-890 :	rrnBT ₁ T ₂ containing HindIII-SspI fragment from pKK223 (Pharmacia)
25	891-3421 :	DraI-EcoRI blunt fragment of pAT ₁₅₃ (Bioexcellence) containing the
30		tetracycline resistance gene and the origin of replication.

Table 5 hereafter corresponds to the complete restriction site analysis of pIGRI.

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Table 5 (cont)

5	Cvi JI	:	190	263	270	380	386	391	421	607	625	714	77
			791	1088	1117	1160	1171	1236	1315	1340	1345	1481	157
			1605	1623	1634	1707	1726	1926	1931	1973	2010	2092	213
10			2157	2162	2300	2310	2329	2370	2427	2435	2465	2478	249
			2544	2588	2732	2748	2804	2822	2886	2894	2932	2946	301
			3087	3122	3245	3269	3388	3403					
15	Cvi QI	:	209	3253									
	Dde I	:	133	336	343	518	608	664	962	1371	1835		
	Dpn I	:	9	236	897	909	987	995	1006	1081	1957	2274	228
			2320	2592	2951	3042	3069						
	Dra II	:	1935	1977	2892								
	Dra III	:	293										
20	Dsa I	:	309	1968	2887								
	Eco 31I	:	562										
	Eco 47III	:	341	1773	2642	2923	3185						
	Eco 57I	:	214										
	Eco 57I*	:	1103										
	Eco 78I	:	2212	2869	2983	3004							
	Eco NI	:	196	2792									
25	Eco RII	:	211	473	583	751	1484	1497	1618	1973	2356	3285	
	Eco RV	:	3232										
	Fnu 4BI	:	378	479	1031	1237	1240	1305	1448	1603	1721	1724	174
			1855	1858	1987	2001	2008	2011	2130	2209	2254	2311	239
30	Fnu DII	:	2479	2644	2695	2802	2836	2839	3117	3120	3191		
		:	489	1021	1602	1784	1881	2003	2029	2174	2184	2313	237
			2440	2445	2472	2601	2716	3072					
	Fok I	:	415	799	3317								
	Fok I*	:	763	2370	2415	3269							
	Gsu I	:	339	2035									
35	Gsu I*	:	2589										
	Hae I	:	775	791	1171	1623	1634	1973	2370	2427	2499		
	Hae II	:	343	541	1405	1775	2214	2644	2871	2925	2985	3006	318
	Hae III	:	625	714	775	791	1171	1605	1623	1634	1973	2157	237
40			2427	2478	2499	2588	2822	2886	2894	3018	3122	3245	
	Hga I	:	158	181	743	2035	2185	2776					
	Hga I*	:	955	1533	2429	2461	3015						
	Hgi AI	:	139	1335	1954	2245	2832	3143					
	Hgi CI	:	208	2126	2210	2649	2867	2981	3002	3296	3339		
	Hgi JII	:	2934	2948									
	Hha I	:	342	489	540	1021	1130	1304	1404	1471	1741	1774	196
45			2000	2062	2213	2472	2603	2643	2718	2870	2924	2984	300
			3158	3186	3318								

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Table 5 (cont)

5	Hin PII	:	340	487	538	1019	1128	1302	1402	1469	1739	1772	196
			1998	2060	2211	2470	2601	2641	2716	2868	2922	2982	300
			3156	3184	3316								
10	Hind II	:	107	371	2766								
	Hind III	:	384	3386									
	Hinf I	:	367	1275	1671	1746	1891	2112	2410	2564	2784		
	Hpa II	:	3	682	716	1077	1267	1293	1440	1932	2133	2159	239
			2487	2647	2723	2883	3006	3015	3030	3247	3256		
15	Hph I	:	94	138	181	663	914	1900	2121	2975	3020	3302	
	Hph I*	:	6										
	Kpn I	:	212										
	Mae I	:	364	899	1152	1928	3187						
	Mae II	:	274	698	944	1847	1871	2460	2516				
	Mae III	:	169	255	304	313	1109	1225	1288	2267	2534	3202	329
20	Mbo I	:	7	234	895	907	985	993	1004	1079	1955	2272	228
			2318	2590	2949	3040	3067						
	Mbo II	:	207	422	917	1779	1827	2419	2690				
	Mbo II*	:	988	2944									
	Mme I*	:	1252	1436	3112	3199							
	Mnl I	:	1218	1542	1948	2446	2630						
25	Mnl I*	:	208	289	337	711	1467	1750	2116	2143	2181	2242	254
			2811	3030	3234	3294							
	Mse I	:	179	186	221	433	764	941	3361	3383	3420		
	Mst I	:	1963	2061	3157								
	Nae I	:	2134	2488	2648	3016							
	Nar I	:	2211	2868	2982	3003							
30	Nco I	:	309										
	Nhe I	:	3186										
	Nla III	:	166	230	313	512	567	859	929	1649	1828	1962	216
			2226	2241	2369	2486	2672	2711	2857	2930	3068	3415	
35	Nla IV	:	210	330	496	1578	1617	1936	1979	2093	2128	2163	221
			2530	2651	2869	2893	2983	3004	3042	3088	3298	3341	
	Nru I	:	2445										
	Nsp BII	:	1062	1307	2278								
	Nsp BI	:	1649	2857									
	Pfl MI	:	293	2052	2101								
	Ple I	:	375	1754									
40	Ple I*	:	1269	2778									
	Ppu MI	:	1935	1977									
	Pss I	:	1938	1980	2895								
	Pst I	:	379										
	Rsa I	:	210	3254									
	Sal I	:	369	2764									
45	Scr PI	:	4	213	475	585	683	716	753	1268	1486	1499	162
			1933	1975	2159	2358	2883	3247	3287				
	Sdu I	:	139	1335	1954	2245	2832	2934	2948	3143			
	Sec I	:	3	309	1485	1968	2046	2248	2881	2887	3286	3300	
	Sfa NI	:	597	765	2392	2767	3178	3291					
50	Sfa NI*	:	1548	1985	2380	3001	3013	3202					

Total number of cuts is : 705.

25	Cvi	JI	:	61	Sdu	I	:	8	Tth111111*	:	3	Ava	I	:	1	
	Fnu	4HI	:	31	Cau	II	:	8	Nsp	BII	:	3	Taq	IIB	:	1
	Bha	I	:	25	Bbv	I	:	8	Fok	I	:	3	Alw	NI	:	1
	Bin	PII	:	25	Mbo	II	:	7	Pfl	MI	:	3	Dra	III	:	1
	Rae	III	:	21	Ava	II	:	7	Hind	II	:	3	Afl	III	:	1
	Nla	IV	:	21	Mae	II	:	7	Dsa	I	:	3	Cla	I	:	1
	Nla	III	:	21	Sfa	NI	:	6	Bsp	HI	:	3	Eco	57I*	:	1
	Hpa	II	:	20	Xho	II	:	6	Pss	I	:	3	Nhe	I	:	1
	Scr	FI	:	18	Hgi	AI	:	6	Mst	I	:	3	Gsu	I*	:	1
30	Sso	II	:	18	Sfa	NI*	:	6	Hgi	JII	:	2	Bal	I	:	1
	Fnu	DII	:	17	Bbv	I*	:	6	Ple	I	:	2	Eco	RV	:	1
	Mbo	I	:	16	Cfr	10I	:	6	Mbo	II*	:	2	Sph	I	:	1
	Dpn	I	:	16	Hga	I	:	6	Cvi	QI	:	2	Xma	III	:	1
	Mnl	I*	:	15	Acy	I	:	5	Acc	I	:	2	Hph	I*	:	1
	Asu	I	:	12	Bin	I	:	5	Bgl	I	:	2	Taq	IIB*	:	1
	Hae	II	:	11	Cfr	I	:	5	Ple	I*	:	2	Eco	57I	:	1
35	Mae	III	:	11	Hga	I*	:	5	Gsu	I	:	2	Kpn	I	:	1
	Rph	I	:	10	Mae	I	:	5	Ppu	MI	:	2	Xba	I	:	1
	Bst	NI	:	10	Eco	47III	:	5	Tth111111	:	2	Aha	III	:	1	
	Eco	RII	:	10	Mnl	I	:	5	Hind	III	:	2	Nru	I	:	1
	Sec	I	:	10	Mme	I*	:	4	Nsp	BI	:	2	Bam	HI	:	1
	Dde	I	:	9	Eco	78I	:	4	Rsa	I	:	2	Apa	LI	:	1
	Binf	I	:	9	Nae	I	:	4	Sal	I	:	2	Asp	718I	:	1
40	Hae	I	:	9	Bbe	I	:	4	Bbv	II	:	2	Eco	31I	:	1
	Alu	I	:	9	Bin	I*	:	4	Bsp	MI	:	2	Nco	I	:	1
	Hgi	CI	:	9	Nar	I	:	4	Sty	I	:	2	Pst	I	:	1
	Mse	I	:	9	Fok	I*	:	4	Eco	NI	:	2				
	Taq	I	:	9	Dra	II	:	3	Xmn	I	:	2				

Aat II , Afl II , Apa I , Asu II , Avr II , Bbv II* , Bcl I ,
Bgl II , Bsp MI* , Bsp MII , Bss HII , Bst EII , Bst XI , Eco 3II* ,

Table 5 (cont)

5 Eco RI , Esp I , Hpa I , Mlu I , Mne I , Nde I , Not I ,
 Nsi I , Pma CI , Pvu I , Pvu II , Rsr II , Sac I , Sac II ,
 Sau I , Sca I , Sci I , Sfi I , Sma I , Sna BI , Spe I ,
 Spl I , Ssp I , Stu I , Taq IIA , Taq IIA* , Tth IIII , Vsp I ,
 Xca I , Xho I , Xma I

10 Total number of selected enzymes which do not cut: 45

Figure 11a corresponds to the restriction and genetic map of the pmTNF MPH plasmid used in Example V for the expression of the P₃₂ antigen of the invention in E. coli.

15 Figure 11b corresponds to the pmTNF-MPH nucleic acid sequence.

On this figure, the origin of nucleotide stretches used to construct plasmid pmTNF-MPH is specified hereafter.

20 Position

1-208 : lambda PL containing EcoRI blunt-MboII
 blunt fragment of pPL(λ) (Pharmacia)

25 209-436 : synthetic DNA fragment

 230-232 : initiation codon (ATG) of mTNF
 fusion protein

30 236-307 : sequence encoding AA 2 to 25 of
 mature mouse TNF

 308-384 : multiple cloning site containing
 His₆ encoding sequence at position
 315-332

35 385-436 : HindIII fragment containing E. coli
 trp terminator

40 437-943 : rrnBT₁T₂ containing HindIII-SspI
 fragment from pKK223 (Pharmacia)

 944-3474 : DraI-EcoRI blunt fragment of pAT₁₅₃
 (Bioexcellence) containing the
 tetracycline resistance gene and the
 origin of replication.

50 Table 6 hereafter corresponds to the complete restriction site analysis of pmTNF-MPH.

 * RESTRICTION-SITE ANALYSIS *

10

Total number of bases is: 3474.
Analysis done on the complete sequence.

List of cuts by enzyme.

Acc I	:	371	2818										
Acy I	:	788	2264	2921	3035	3056							
Afl II	:	387											
Afl III	:	1698											
Aha III	:	224											
Alu I	:	386	439	1141	1398	1534	1760	2382	2785	3441	3456		
Alw NI	:	1289											
Apa I	:	345											
Apa LI	:	1384											
Asp 718I	:	210											
Asu I	:	341	342	547	676	766	1988	2030	2209	2333	2582	267	
		2945	3297										
Ava I	:	338	2043										
Ava II	:	547	1988	2030	2333	2582	2670						
Bal I	:	2026											
Bam HI	:	334	3093										
Bbe I	:	2267	2924	3038	3059								
Bbv I	:	1369	1788	1806	1919	1922	2866	3255					
Bbv I*	:	1070	1276	1279	2026	2050	2683						
Bbv II	:	1875	2738										
Bgl I	:	2306	2540										
Bin I	:	17	342	956	1054	1140	3101						
Bin I*	:	329	955	1052	2366	3088							
Bsp HI	:	908	978	2979									
Bsp MI	:	2414											
Bsp MII	:	354											
Bst NI	:	215	528	638	806	1539	1552	1673	2028	2411	3340		
Cau II	:	6	339	340	736	769	1321	1986	2212	2936	3300		
Cfr 10I	:	374	2185	2539	2699	3058	3067	3308					
Cfr I	:	2024	2529	2937	3069	3173							
Cla I	:	3446											
Cvi JI	:	192	265	272	343	350	361	386	400	439	444	47	
		660	678	767	828	844	1141	1170	1213	1224	1289	136	
		1393	1398	1534	1632	1658	1676	1687	1760	1779	1979	198	
		2026	2063	2145	2189	2210	2215	2353	2363	2382	2423	248	

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Table 6 (cont)

5			2488	2518	2531	2552	2597	2641	2785	2801	2857	2875	293
			2947	2985	2999	3071	3140	3175	3298	3322	3441	3456	
	Cvi QI	:	211	3306									
	Dde I	:	135	571	661	717	1015	1424	1888				
10	Dpn I	:	11	238	336	950	962	1040	1048	1059	1134	2010	232
			2342	2373	2645	3004	3095	3122					
	Dra II	:	1988	2030	2945								
	Dra III	:	295	331									
	Dsa I	:	345	2021	2940								
15	Eco 31I	:	615										
	Eco 47III	:	1826	2695	2976	3238							
	Eco 57I	:	216										
	Eco 57I*	:	1156										
	Eco 78I	:	2265	2922	3036	3057							
	Eco NI	:	198	2845									
	Eco RI	:	309										
20	Eco RII	:	213	526	636	804	1537	1550	1671	2026	2409	3338	
	Eco RV	:	3285										
	Fnu 48I	:	401	417	532	1084	1290	1293	1358	1501	1656	1774	177
			1795	1908	1911	2040	2054	2061	2064	2183	2262	2307	236
25	Fnu DII	:	2447	2532	2697	2748	2855	2889	2892	3170	3173	3244	
			542	1074	1655	1837	1934	2056	2082	2227	2237	2366	243
			2493	2498	2525	2654	2769	3125					
	Fok I	:	468	852	3370								
	Fok I*	:	816	2423	2468	3322							
	Gsu I	:	2088										
30	Gsu I*	:	2642										
	Hae I	:	361	828	844	1224	1676	1687	2026	2423	2480	2552	
	Hae II	:	594	1458	1828	2267	2697	2924	2978	3038	3059	3240	
	Hae III	:	343	361	678	767	828	844	1224	1658	1676	1687	202
			2210	2423	2480	2531	2552	2641	2875	2939	2947	3071	317
35	Hga I	:	3298										
	Hga I*	:	160	183	796	2088	2238	2829					
	Hgi AI	:	1008	1586	2482	2514	3068						
	Hgi CI	:	141	1388	2007	2298	2885	3196					
	Hgi JII	:	210	2179	2263	2702	2920	3034	3055	3349	3392		
	Hha I	:	345	2987	3001								
40			542	593	1074	1183	1357	1457	1524	1794	1827	2017	205
			2115	2266	2525	2656	2696	2771	2923	2977	3037	3058	321
	Hin PII	:	3239	3371									
			540	591	1072	1181	1355	1455	1522	1792	1825	2015	205
			2113	2264	2523	2654	2694	2769	2921	2975	3035	3056	320
45			3237	3369									
	Hind II	:	109	372	2819								
	Hind III	:	384	437	3439								
	Hinf I	:	368	1328	1724	1799	1944	2165	2463	2617	2837		

Table 6 (cont)

5	Hpa II	:	5	339	355	375	735	769	1130	1320	1346	1493	198
			2186	2212	2450	2540	2700	2776	2936	3059	3068	3083	330
			3309										
10	Hph I	:	96	140	183	716	967	1953	2174	3028	3073	3355	
	Hph I*	:	8	305	311	317							
	Kpn I	:	214										
	Mae I	:	365	952	1205	1981	3240						
	Mae II	:	276	330	751	997	1900	1924	2513	2569			
	Mae III	:	171	257	1162	1278	1341	2320	2587	3255	3343		
	Mbo I	:	9	236	334	948	960	1038	1046	1057	1132	2008	232
15			2340	2371	2643	3002	3093	3120					
	Mbo II	:	209	475	970	1832	1880	2472	2743				
	Mbo II*	:	1041	2997									
	Mne I*	:	1305	1489	3165	3252							
	Mnl I	:	372	1271	1595	2001	2499	2683					
20	Mnl I*	:	210	291	350	764	1520	1803	2169	2196	2234	2295	259
			2864	3083	3287	3347							
	Mse I	:	181	188	223	388	486	817	994	3414	3436		
	Mst I	:	2016	2114	3210								
	Nae I	:	2187	2541	2701	3069							
	Nar I	:	2264	2921	3035	3056							
25	Nco I	:	345										
	Nhe I	:	3239										
	Nla III	:	168	232	349	382	565	620	912	982	1702	1881	201
			2222	2279	2294	2422	2539	2725	2764	2910	2983	3121	346
30	Nla IV	:	212	336	343	549	1631	1670	1989	2032	2146	2181	221
			2265	2583	2704	2922	2946	3036	3057	3095	3141	3351	339
	Nru I	:	2498										
	Nsp BII	:	412	1115	1360	2331							
	Nsp BI	:	382	1702	2910								
35	Pfl MI	:	295	2105	2154								
	Ple I	:	376	1807									
	Ple I*	:	1322	2831									
	Pma CI	:	331										
	Ppu MI	:	1988	2030									
	Pss I	:	1991	2033	2948								
	Rsa I	:	212	3307									
40	Sal I	:	370	2817									
	Scr FI	:	6	215	339	340	528	638	736	769	806	1321	153
			1552	1673	1986	2028	2212	2411	2936	3300	3340		
	Sdu I	:	141	345	1388	2007	2298	2885	2987	3001	3196		
	Sec I	:	5	338	345	1538	2021	2099	2301	2934	2940	3339	335
45	Sfa NI	:	650	818	2445	2820	3231	3344					
	Sfa NI*	:	420	1601	2038	2433	3054	3066	3255				
	Sma I	:	340										
	Sph I	:	382	2910									
	Sso II	:	4	213	337	338	526	636	734	767	804	1319	153

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Table 6 (con't)

5			1550	1671	1984	2026	2210	2409	2934	3298	3338
	Stu I	:	361								
	Sty I	:	345	2099							
	Taq I	:	254	371	666	1600	2202	2343	2818	3131	3446
	Taq IIB	:	1802								
	Taq IIB*	:	2804								
10	Tth11111	:	40	1107							
	Tth11111*	:	686	1075	1114						
	Xba I	:	364								
	Xho II	:	9	334	948	960	1046	1057	3093		
	Xma I	:	338								
	Xma III	:	2529								
15	Xmn I	:	1	467							

Total number of cuts is : 743.

20

25 List of non cutting selected enzymes.

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Aat II	, Asu II	, Avr II	, Bbv II*	, Bcl I	, Bgl II	, Bsp MI*	,
Bss HII	, Bst BII	, Bst XI	, Eco 31I*	, Esp I	, Hpa I	, Mlu I	,
Mme I	, Nde I	, Not I	, Nsi I	, Pst I	, Pvu I	, Pvu II	,
Rsr II	, Sac I	, Sac II	, Sau I	, Sca I	, Sci I	, Sfi I	,
30 Sna BI	, Spe I	, Spl I'	, Ssp I	, Taq IIA	, Taq IIA*	, Tth 1111	,
Vsp I	, Xca I	, Xho I					

Total number of selected enzymes which do not cut: 38

35

Figure 12a corresponds to the restriction and genetic map of the plasmid pIG2 used to make the intermediary construct PIGS Mt32 as described in Example IV for the subcloning of the P₃₂ antigen in plasmid pIGRI.

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Figure 12b corresponds to the pIG2 nucleic acid sequence.

On this figure, the origin of nucleotide stretches used to construct plasmid pIG2 is specified hereafter.

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Position

5	3300-206 :	lambda PL containing EcoRI-MboII blunt fragment of pPL(λ) (Pharmacia)
10	207-266 :	synthetic sequence containing multiple cloning site and ribosome binding site of which the ATG initiation codon is located at position 232-234
15	267-772 :	rrnBT ₁ T ₂ containing HindIII-SspI fragment from pKK223 (Pharmacia)
20	773-3300 :	tetracycline resistance gene and origin of replication containing EcoRI-DraI fragment of pAT 153 (Bioexcellence)

Table 7 corresponds to the complete restriction site analysis of pIG2.

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Table 9

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 * RESTRICTION-SITE ANALYSIS *

Done on DNA sequence pIG2

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Total number of bases is: 3301.
 Analysis done on the complete sequence.

15

List of cuts by enzyme.
 =====

	Acc I	:	252	2647															
	Acy I	:	617	2093	2750	2864	2885												
	Afl III	:	1527																
	Aha III	:	222																
20	Alu I	:	268	970	1227	1363	1589	2211	2614	3270	3285								
	Alw NI	:	1118																
	Apa LI	:	1213																
	Asp 718I	:	208																
	Asu I	:	376	505	595	1817	1859	2038	2162	2411	2499	2774	312						
	Ava I	:	1872																
	Ava II	:	376	1817	1859	2162	2411	2499											
25	Bal I	:	1855																
	Bam HI	:	239	2922															
	Bbe I	:	2096	2753	2867	2888													
	Bbv I	:	271	1198	1617	1635	1748	1751	2695	3084									
	Bbv I*	:	899	1105	1108	1855	1879	2512											
	Bbv II	:	1704	2567															
30	Bgl I	:	2135	2369															
	Bin I	:	15	247	785	883	969	2930											
	Bin I*	:	234	784	881	2195	2917												
	Bsp HI	:	737	807	2808														
	Bsp MI	:	264	2243															
	Bst NI	:	213	357	467	635	1368	1381	1502	1857	2240	3169							
	Cau II	:	4	565	598	1150	1815	2041	2765	3129									
35	Cfr 10I	:	2014	2368	2528	2887	2896	3137											
	Cfr I	:	1853	2358	2766	2898	3002												
	Cla I	:	3275																
	Cvi JI	:	190	262	268	273	303	489	507	596	657	673	97						
			999	1042	1053	1118	1197	1222	1227	1363	1461	1487	150						
			1516	1589	1608	1808	1813	1855	1892	1974	2018	2039	204						
			2182	2192	2211	2252	2309	2317	2347	2360	2381	2426	247						
40			2614	2630	2686	2704	2768	2776	2814	2828	2900	2969	300						
			3127	3151	3270	3285													
	Cvi QI	:	209	3135															
	Dde I	:	133	400	490	546	844	1253	1717										
	Dpn I	:	9	241	779	791	869	877	888	963	1839	2156	217						
			2202	2474	2833	2924	2951												
45	Dra II	:	1817	1859	2774														
	Dsa I	:	230	1850	2769														
	Eco 31I	:	444																
	Eco 47III	:	1655	2524	2805	3067													
	Eco 57I	:	214																
	Eco 57I*	:	985																

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	Eco 78I	:	2094	2751	2865	2886							
	Eco NI	:	196	2674									
	Eco RII	:	211	355	465	633	1366	1379	1500	1855	2238	3167	
	Eco RV	:	3114										
10	Fnu 4HI	:	260	361	913	1119	1122	1187	1330	1485	1603	1606	162
			1737	1740	1869	1883	1890	1893	2012	2091	2136	2193	227
			2361	2526	2577	2684	2718	2721	2999	3002	3073		
	Fnu DII	:	371	903	1484	1666	1763	1885	1911	2056	2066	2195	226
			2322	2327	2354	2483	2598	2954					
	Fok I	:	297	681	3199								
	Fok I*	:	645	2252	2297	3151							
15	Gsu I	:	1917										
	Gsu I*	:	2471										
	Hae I	:	657	673	1053	1505	1516	1855	2252	2309	2381		
	Hae II	:	423	1287	1657	2096	2526	2753	2807	2867	2888	3069	
	Hae III	:	507	596	657	673	1053	1487	1505	1516	1855	2039	225
			2309	2360	2381	2470	2704	2768	2776	2900	3004	3127	
20	Hga I	:	158	181	625	1917	2067	2658					
	Hga I*	:	837	1415	2311	2343	2897						
	Bgi AI	:	139	1217	1836	2127	2714	3025					
	Bgi CI	:	208	2008	2092	2531	2749	2863	2884	3178	3221		
	Bgi JII	:	2816	2830									
	Bha I	:	371	422	903	1012	1186	1286	1353	1623	1656	1846	188
			1944	2095	2354	2485	2525	2600	2752	2806	2866	2887	304
25			3068	3200									
	Bin PII	:	369	420	901	1010	1184	1284	1351	1621	1654	1844	188
			1942	2093	2352	2483	2523	2598	2750	2804	2864	2885	303
			3066	3198									
	Bind II	:	107	253	2648								
	Bind III	:	266	3268									
	Binf I	:	249	1157	1553	1628	1773	1994	2292	2446	2666		
30	Bpa II	:	3	564	598	959	1149	1175	1322	1814	2015	2041	227
			2369	2529	2605	2765	2888	2897	2912	3129	3138		
	Bph I	:	94	138	181	545	796	1782	2003	2857	2902	3184	
	Bph I*	:	6										
	Kpn I	:	212										
	Mae I	:	246	781	1034	1810	3069						
	Mae II	:	580	826	1729	1753	2342	2398					
35	Mae III	:	169	991	1107	1170	2149	2416	3084	3172			
	Mbo I	:	7	239	777	789	867	875	886	961	1837	2154	216
			2200	2472	2831	2922	2949						
	Mbo II	:	207	304	799	1661	1709	2301	2572				
	Mbo II*	:	870	2826									
	Mme I*	:	1134	1318	29								

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11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040 1041 1042 1043 1044 10

On this figure :

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(---)

represents the polylinker sequence,
- the double underlined sequence

(=====)

represents the extra amino acids created at cloning site, and

- the amino acid marked with nothing is the antigen sequence starting from the amino acid at position 4 of figure 5.

Figure 14a and 14b correspond to the expression of the mTNF-His₆-P₃₂ fusion protein in K12ΔH, given in Example VI, with Fig. 14a representing the Coomassie Brilliant Blue stained SDS-PAGE and 14b representing immunoblots of the gel with anti-32-kDa and anti-mTNF-antibody.

On fig. 14a, the lanes correspond to the following:

Lanes	
1.	protein molecular weight markers
2.	pmTNF-MPH-Mt32 28 °C 1 h induction
3.	pmTNF-MPH-Mt32 42 °C 1 h induction
4.	pmTNF-MPH-Mt32 42 °C 2 h induction
5.	pmTNF-MPH-Mt32 42 °C 3 h induction
6.	pmTNF-MPH-Mt32 28 °C 4 h induction
7.	pmTNF-MPH-Mt32 42 °C 4 h induction
8.	pmTNF-MPH-Mt32 28 °C 5 h induction
9.	pmTNF-MPH-Mt32 42 °C 5 h induction

On fig. 14b, the lanes correspond to the following:

Lanes	
1.	pmTNF-MPH-Mt32 28 °C 1 h induction
2.	pmTNF-MPH-Mt32 42 °C 1 h induction
3.	pmTNF-MPH-Mt32 28 °C 4 h induction
4.	pmTNF-MPH-Mt32 42 °C 4 h induction

Figure 15 corresponds to the IMAC elution profile of the recombinant antigen with decreasing pH as presented in Example VII.

Figure 16 corresponds to the IMAC elution profile of the recombinant antigen with increasing imidazole concentrations as presented in Example VII.

Figure 17 corresponds to the IMAC elution profile of the recombinant antigen with a step gradient of increasing imidazole concentrations as presented in Example VII.

EXAMPLE I:

MATERIAL AND METHODS

Screening of the λgt11 M. tuberculosis recombinant DNA library with anti-32-kDa antiserum

A λgt11 recombinant library constructed from genomic DNA of *M. tuberculosis* (Erdman strain) was obtained from R. Young (35). Screening was performed as described (14,35) with some modifications hereafter mentioned. λgt11 infected *E. coli* Y1090 (10⁵ pfu per 150 mm plate) were seeded on NZYM plates (Gibco)(16) and incubated at 42 °C for 24 hrs. To induce expression of the β-galactosidase-fusion proteins the plates were overlaid with isopropyl β-D-thiogalactoside (IPTG)-saturated filters (Hybond C extra, Amersham), and incubated for 2 hrs at 37 °C. Screening was done with a polyclonal rabbit anti-32-kDa antiserum. Said polyclonal antiserum rabbit anti-32-kDa antiserum was obtained by raising antiserum

against the P₃₂ *M. bovis* BCG (strain 1173P2 - Institut Pasteur Paris) as follows: 400 µg (purified P₃₂ protein of *M. bovis* BCG) per ml physiological saline were mixed with one volume of incomplete Freund's adjuvant. The material was homogenized and injected intradermally in 50 µl doses, delivered at 10 sites in the back of the rabbits, at 0, 4, 7 and 8 weeks (adjuvant was replaced by the diluent for the last injection).

5 One week later, the rabbits were bled and the sera tested for antibody level before being distributed in aliquots and stored at -80 °C.

The polyclonal rabbit anti-32-kDa antiserum was pre-absorbed on *E. coli* lysate (14) and used at a final dilution of 1:300. A secondary alkaline-phosphatase anti-rabbit IgG conjugate (Promega), diluted at 1:5000 was used to detect the β -galactosidase fusion proteins. For color development nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used. Reactive areas on the filter turned deep purple within 30 min. Usually three consecutive purification steps were performed to obtain pure clones. IPTG, BCIP and NBT were from Promega corp. (Madison WI.).

10

15 Plaque screening by hybridization for obtaining the secondary clones BY1, By2 and By5 hereafter defined

The procedure used was as described by Maniatis et al. (14).

20 Preparation of crude lysates from λ gt11 recombinant lysogens

Colonies of *E. coli* Y1089 were lysogenized with appropriate λ gt11 recombinants as described by Hyunh et al. (14). Single colonies of lysogenized *E. coli* Y1089 were inoculated into LB medium and grown to an optical density of 0.5 at 600nm at 30 °C. After a heat shock at 45 °C for 20 min., the production of β -galactosidase-fusion protein was induced by the addition of IPTG to a final concentration of 10 mM.

25 Incubation was continued for 60 min. at 37 °C and cells were quickly harvested by centrifugation. Cells were concentrated 50 times in buffer (10 mM Tris pH 8.0, 2 mM EDTA) and rapidly frozen into liquid nitrogen. The samples were lysed by thawing and treated with 100 µg/ml DNase I in EcoRI restriction buffer, for 5-10 minutes at 37 °C.

30

Immunoblotting (Western blotting) analysis:

After SDS-PAGE electrophoresis, recombinant lysogen proteins were blotted onto nitrocellulose membranes (Hybond C, Amersham) as described by Towbin et al. (29). The expression of mycobacterial antigens, fused to β -galactosidase in *E. coli* Y1089 was visualized by the binding of a polyclonal rabbit anti-32-kDa antiserum (1:1000) obtained as described in the above paragraph "Screening of the λ gt11 *M. tuberculosis* recombinant DNA library with anti-32-kDa antiserum" and using a monoclonal anti- β -galactosidase antibody (Promega). A secondary alkaline-phosphatase anti-rabbit IgG conjugate (Promega) diluted at 1:5000, was used to detect the fusion proteins.

40

The use of these various antibodies enables to detect the β -galactosidase fusion protein. This reaction is due to the *M. tuberculosis* protein because of the fact that non fused- β -galactosidase is also present on the same gel and is not recognized by the serum from tuberculous patients.

In order to identify selective recognition of recombinant fusion proteins by human tuberculous sera, nitrocellulose sheets were incubated overnight with these sera (1:50) (after blocking aspecific protein binding sites). The human tuberculous sera were selected for their reactivity (high or low) against the purified 32-kDa antigen of *M. bovis* BCG tested in a Dot blot assay as previously described (31). Reactive areas on the nitrocellulose sheets were revealed by incubation with peroxidase conjugated goat anti-human IgG antibody (Dakopatts, Copenhagen, Denmark) (1:200) for 4 hrs and after repeated washings color reaction was developed by adding peroxidase substrate (α -chloronaphtol) (Bio-Rad) in the presence of peroxidase and hydrogen peroxide.

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Recombinant DNA analysis

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Initial identification of *M. tuberculosis* DNA inserts in purified λ gt11 clones was performed by EcoRI restriction. After digestion, the excised inserts were run on agarose gels and submitted to Southern hybridization. Probes were labeled with α ³²P-dCTP by random priming (10). Other restriction sites were

located by single and double digestions of recombinant λ gt11 phage DNA or their subcloned EcoRI fragments by HindIII, pstI, KpnI, AccI and SphI.

5 Sequencing

Sequence analysis was done by the primer extension dideoxy termination method of Sanger et al. (25) after subcloning of specific fragments in Bluescribe-M13 (6) or in mp10 and mp11 M13 vectors (Methods in Enzymology, vol. 101, 1983, p.20-89, Joachim Messing, New M13 vectors for cloning, Academic Press).
 10 Sequence analysis was greatly hampered by the high GC content of the *M. tuberculosis* DNA (65%). Sequencing reactions were therefore performed with several DNA polymerases: T7 DNA polymerase ("Sequenase" USB), Klenow fragment of DNA polymerase I (Amersham) and in some cases with AMV reverse transcriptase (Super RT, Anglian Biotechnology Ltd.) and sometimes with dITP instead of dGTP. Several oligodeoxynucleotides were synthesized and used to focus ambiguous regions of the sequence.
 15 The sequencing strategy is summarized in Fig. 2 In order to trace possible artefactual frameshifts in some ambiguous regions, a special program was used to define the most probable open reading frame in sequences containing a high proportion of GC (3). Several regions particularly prone to sequencing artefacts were confirmed or corrected by chemical sequencing (18). For this purpose, fragments were subcloned in the chemical sequencing vector pGV462 (21) and analysed as described previously. Selected restriction
 20 fragments of about 250-350bp were isolated, made blunt-ended by treatment with either Klenow polymerase or Mung bean nuclease, and subcloned in the SmaI or HincII site of pGV462. Both strands of the inserted DNA were sequenced by single-end labeling at Tth 111I or BstEII (32) and a modified chemical degradation strategy (33).

Routine computer aided analysis of the nucleic acid and deduced amino acid sequences were
 25 performed with the LGBC program from Bellon (2). Homology searches used the FASTA programs from Pearson and Lipman (23) and the Protein Identification Resource (PIR) from the National Biomedical Research Foundation -Washington (NBRF) (NBRF/PIR data bank), release 16 (march 1988).

30 RESULTS

- Screening of the λ gt11M. *M. tuberculosis* recombinant DNA library with polyclonal anti-32-kDa antiserum :

35 Ten filters representing 1.5×10^6 plaques were probed with a polyclonal rabbit anti-32-kDa antiserum (8). Following purification, six independent positive clones were obtained.

Analysis of recombinant clones

40 EcoRI restriction analysis of these 6 purified λ gt11 recombinant clones DNA, (Fig. 1A) revealed 4 different types of insert. Clone 15 had an insert with a total length of 3.8 kb with two additional internal EcoRI sites resulting in three DNA fragments of 1.8 kb, 1.5 kb and 0.5 kb. The DNA Insert of clone 16 was 1.7 kb long. Clones 17 and 19 had a DNA insert of almost identical length being 2.7 kb and 2.8 kb
 45 respectively.

Finally, clone 23 (not shown) and clone 24 both contained an insert of 4 kb with one additional EcoRI restriction site giving two fragments of 2.3 kb and 1.7 kb. Southern analysis (data not shown) showed that the DNA inserts of clones 15, 16, 19 and the small fragment (1.7 kb) of clone 24 only hybridized with themselves whereas clone 17 (2.7 kb) hybridized with itself but also equally well with the 2.3 kb DNA
 50 fragment of clone 24. Clones 15, 16 and 19 are thus distinct and unrelated to the 17, 23, 24 group. This interpretation was further confirmed by analysis of crude lysates of *E. coli* Y1089 lysogenized with the appropriate λ gt11 recombinants and induced with IPTG. Western blot analysis (Fig. 1B), showed no fusion protein, either mature or incomplete, reactive with the polyclonal anti-32-kDa antiserum in cells expressing clones 15, 16 and 19. Clones 15, 16 and 19, were thus considered as false positives and were not further
 55 studied. On the contrary, cells lysogenized with clone 23 and 24 produced an immunoreactive fusion protein containing about 10 kDa of the 32-kDa protein. Clone 17 finally expressed a fusion protein of which the foreign polypeptide part is about 25 kDa long. The restriction endonuclease maps of the 2.3 kb insert of clone 24 and of the 2.7 kb fragment of clone 17 (Fig. 2) allowed us to align and orient the two inserts

suggesting that the latter corresponds to a ± 0.5 kb 5' extension of the first.

As clone 17 was incomplete, the same λ gt11 recombinant *M. tuberculosis* DNA library was screened by hybridization with a 120 bp EcoRI-Kpn1 restriction fragment corresponding to the very 5' end of the DNA insert of clone 17 (previously subcloned in a Blue Scribe vector commercialized by Vector cloning Systems (Stratagene Cloning System) (Fig. 2). Three 5'-extended clones By1, By2 and By5 were isolated, analyzed by restriction and aligned. The largest insert, By5 contained the information for the entire coding region (see below) flanked by 3.1 kb upstream and 1.1 kb downstream (Fig. 2).

10 DNA sequencing

The 1358 base pairs nucleotide sequence derived from the various λ gt11 overlapping clones is represented in Fig. 3a and Fig. 3b. The DNA sequence contains a 1059 base pair open reading frame starting at position 183 and ending with a TAG codon at position 1242. It occurs that the NH₂-terminal amino-acid sequence, (phe-ser-arg-pro-gly-leu-pro-val-glu-tyr-leu-gln-val-pro-ser-pro-ser-met-gly-arg-asp-ile-lys-val-gln-phe-gln-ser-gly-gly-ala-asn) which can be located within this open reading frame from the nucleotide sequence beginning with a TTT codon at position 360 corresponds to the same NH₂-terminal amino acid sequence of the MPB 59 antigen except for the amino acids at position 20, 21, 31, which are respectively gly, cys and asn in the MPB 59 (34). Therefore, the DNA region upstream of this sequence is expected to encode a signal peptide required for the secretion of a protein of 32-kDa. The mature protein thus presumably consists of 295 amino acid residues from the N-terminal Phe (TTT codon) to the C-terminal Ala (GCC codon) (Fig. 5).

Six ATG codons were found to precede the TTT at position 360 in the same reading frame. Usage of any of these ATGs in the same reading frame would lead to the synthesis of signal peptides of 29,42,47,49,55 and 59 residues.

Hydropathy pattern

The hydropathy pattern coding sequence of the protein of 32-kDa of the invention and that of the antigen α of BCG (17) were determined by the method of Kyte and Doolittle (15). The nonapeptide profiles are shown in Fig. 6. Besides the initial hydrophobic signal peptide region, several hydrophilic domains could be identified. It is interesting to note that the overall hydrophilicity pattern of the protein of 32-kDa of the invention is comparable to that of the antigen α . For both proteins, a domain of highest hydrophilicity could be identified between amino acid residues 200 and 250.

Homology

Matsuo et al. (17) recently published the sequence of a 1095 nucleotide cloned DNA corresponding to the gene coding for the antigen α of BCG. The 978 bp coding region of *M. bovis* antigen α as revised in Infection and Immunity, vol. 58, p. 550-556, 1990, and 1017 bp coding regions of the protein of 32-kDa of the invention show a 77.5% homology, in an aligned region of 942 bp. At the amino acid level both precursor protein sequences share 75.6% identical residues. In addition, 17.6% of the amino acids correspond to evolutionary conserved replacements as defined in the algorithm used for the comparison (PAM250 matrix, ref 23). Figure 7 shows sequence divergences in the N-terminal of the signal peptide. The amino terminal sequence - 32 amino acids - of both mature proteins is identical except for position 31.

50 Human sera recognize the recombinant 32-kDa protein

Fig. 8 shows that serum samples from tuberculous patients when immunoblotted with a crude *E. coli* extract expressing clone 17 distinctly react with the 140 kDa fusion protein (lanes 4 to 6) contain the protein of 32-kDa of the invention, but not with unfused β -galactosidase expressed in a parallel extract (lanes 10 to 12). Serum samples from two negative controls selected as responding very little to the purified protein of 32-kDa of the invention does neither recognize the 140 kDa fused protein containing the protein of 32-kDa of the invention, nor the unfused β -galactosidase (lanes 2, 3 and 8 and 9). The 140 kDa fused protein and the unfused β -galactosidase were easily localized reacting with the anti- β -galactosidase monoclonal

antibody (lanes 1 to 7).

The invention has enabled to prepare a DNA region coding particularly for a protein of 32-kDa (cf. fig.5); said DNA region containing an open reading frame of 338 codons (stop codon non included). At position 220 a TTT encoding the first amino acid of the mature protein is followed by the 295 triplets coding for the mature protein of 32-kDa. The size of this open reading frame, the immunoreactivity of the derived fusion proteins, the presence of a signal peptide and, especially, the identification within this gene of a NH₂-terminal region highly homologous to that found in the MPB 59 antigen (31/32 amino acids homology) and in the BCG antigen α (31/32 amino acids homology) (see Fig. 7), strongly suggest that the DNA fragment described contains the complete cistron encoding the protein of 32-kDa secreted by *M. tuberculosis*, which had never been so far identified in a non-ambiguous way.

Six ATG codons were found to precede this TTT at position 220 in the same reading frame. Usage of any of these ATGs in the same reading frame would lead to the synthesis of signal peptides of 43, 48, 50, 56 or 60 residues. Among these various possibilities, initiation is more likely to take place either at ATG₉₁ or ATG₅₂ because both are preceded by a plausible *E. coli* -like promoter and a Shine-Dalgarno motif.

If initiation takes place at ATG₉₁, the corresponding signal peptide would code for a rather long peptide signal of 43 residues. This length however is not uncommon among secreted proteins from Gram positive bacteria (5). It would be preceded by a typical *E. coli* Shine-Dalgarno motif (4/6 residues homologous to AGGAGG) at a suitable distance.

If initiation takes place at ATG₅₂, the corresponding signal peptide would code for a peptide signal of 56 residues but would have a less stringent Shine-Dalgarno ribosome binding site sequence.

The region encompassing the translation termination triplet was particularly sensitive to secondary structure effects which lead to so-called compressions on the sequencing gels. In front of the TAG termination codon at position 1105, 22 out of 23 residues are G-C base pairs, of which 9 are G's.

Upstream ATG₁₃₀, a sequence resembling an *E. coli* promoter (11) comprising an hexanucleotide (TTGAGA) (homology 5/6 to TTGACA) and a AAGAAT box (homology 4/6 to TATAAT) separated by 16 nucleotides was observed. Upstream the potential initiating codon ATG₉₁, one could detect several sequences homologous to the *E. coli* "-35 hexanucleotide box", followed by a sequence resembling a TATAAT box. Among these, the most suggestive is illustrated on Fig. 3a and 3b. It comprises a TTGGCC at position 59 (fig. 3a and 3b) (homology 4/6 to TTGACA) separated by 14 nucleotides from a GATAAG (homology 4/6 to TATAAT). Interestingly this putative promoter region shares no extensive sequence homology with the promoter region described for the BCG protein α -gene (17) nor with that described for the 65 kDa protein gene (26, 28).

Searching the NBRF data bank (issue 16.0) any significant homology between the protein of 32-kDa of the invention and any other completely known protein sequence could not be detected. In particular no significant homology was observed between the 32-kDa protein and α and β subunits of the human fibronectin receptor (1). The NH₂-terminal sequence of the 32-kDa protein of the invention is highly homologous - 29/32 amino acids - to that previously published for BCG MPB 59 antigen (34) and to that of BCG α -antigen - 31/32 amino acids - (Matsuo, 17) and is identical in its first 6 amino acids with the 32-kDa protein of *M. bovis* BCG (9). However, the presumed initiating methionine precedes an additional 29 or 42 amino acid hydrophobic sequence which differs from the one of α -antigen (cf. Fig. 7), but displaying all the characteristics attributed to signal sequences of secreted polypeptides in prokaryotes (22).

Interestingly, no significant homology between the nucleic acid (1-1358) of the invention (cf. fig. 3a and 3b) and the DNA of the antigen α of Matsuo exists within their putative promoter regions.

EXAMPLE II : CONSTRUCTION OF A BACTERIAL PLASMID CONTAINING THE ENTIRE CODING SEQUENCE OF THE 32-kDa PROTEIN OF *M. TUBERCULOSIS*

In the previous example, in figure 2, the various overlapping λ gt11 isolates covering the 32-kDa protein gene region from *M. tuberculosis* were described. Several DNA fragments were subcloned from these λ gt11 phages in the Blue Scribe M13+ plasmid (Stratagene). Since none of these plasmids contained the entire coding sequence of the 32-kDa protein gene, a plasmid containing this sequence was reconstructed.

Step 1 : Preparation of the DNA fragments :

- 1) The plasmid BS-By5-800 obtained by subcloning HindIII fragments of By5 (cf. fig. 2) into the Blue Scribe M13+ plasmid (Stratagene), was digested with HindIII and a fragment of 800 bp was obtained and

isolated from a 1% agarose gel by electroelution.

2) The plasmid BS-4.1 obtained by subcloning the 2,7 kb EcoRI insert from λ gt11-17) into the Blue Scribe M13⁺ plasmid (Stratagene) (see fig .2 of patent application) was digested with HindIII and SphI and a fragment of 1500 bp was obtained and isolated from a 1% agarose gel by electroelution.

5 3) Blue Scribe M13⁺ was digested with HindIII and SphI, and treated with calf intestine alkaline phosphatase (special quality for molecular biology, Boehringer Mannheim) as indicated by the manufacturer.

Step 2 : ligation :

10

The ligation reaction contained :

125 ng of the 800 bp HindIII fragment (1)

125 ng of the 1500 bp SphI-HindIII insert (2)

50 ng of the HindIII-SphI digested BSM13⁺ vector (3)

15 2 μ l of 10 ligation buffer (Maniatis et al., 1982)

1 μ l of (= 2,5 U) of T4 DNA ligase (Amersham)

4 μ l PEG 6000, 25% (w/v)

8 μ l H₂O

The incubation was for 4 hours at 16° C.

20

Step 3 : Transformation :

100 μ l of DH5 α *E. coli* (Gibco BRL) were transformed with 10 μ l of the ligation reaction (step 2) and
25 plated on IPTG, X-Gal ampicillin plates, as indicated by the manufacturer. About 70 white colonies were obtained.

step 4 :

30

As the 800 bp fragment could have been inserted in both orientations, plasmidic DNA from several clones were analyzed by digestion with PstI in order to select one clone (different from clone 11), characterized by the presence of 2 small fragments of 229 and 294 bp. This construction contains the HindIII-HindIII-SphI complex in the correct orientation. The plasmid containing this new construction was
35 called : "BS.BK.P₃₂.complet".

EXAMPLE III : EXPRESSION OF A POLYPEPTIDE OF THE INVENTION IN E. COLI:

40

The DNA sequence coding for a polypeptide, or part of it, can be linked to a ribosome binding site which is part of the expression vector, or can be fused to the information of another protein or peptide already present on the expression vector.

In the former case the information is expressed as such and hence devoid of any foreign sequences (except maybe for the aminoterminal methionine which is not always removed by *E. coli*).

45

In the latter case the expressed protein is a hybrid or a fusion protein.

The gene, coding for the polypeptide, and the expression vector are treated with the appropriate restriction enzyme(s) or manipulated otherwise as to create termini allowing ligation. The resulting recombinant vector is used to transform a host. The transformants are analyzed for the presence and proper orientation of the inserted gene. In addition, the cloning vector may be used to transform other strains of
50 a chosen host. Various methods and materials for preparing recombinant vectors, transforming them to host cells and expressing polypeptides and proteins are described by Panayatos, N., in "Plasmids, a practical approach (ed. K.G. Hardy, IRL Press) pp.163-176, by Old and Primrose, principals of gene manipulation (2d Ed, 1981) and are well known by those skilled in the art.

Various cloning vectors may be utilized for expression. Although a plasmid is preferable, the vector may
55 be a bacteriophage or cosmid. The vector chosen should be compatible with the host cell chosen.

Moreover, the plasmid should have a phenotypic property that will enable the transformed host cells to be readily identified and separated from those which are not transformed. Such selection genes can be a gene providing resistance to an antibiotic like for instance, tetracyclin, carbenicillin, kanamycin, chloram-

phenicol, streptomycin, etc.

In order to express the coding sequence of a gene in E. coli the expression vector should also contain the necessary signals for transcription and translation.

Hence it should contain a promoter, synthetic or derived from a natural source, which is functional in E. coli. Preferably, although usually not absolutely necessary, the promoter should be controllable by the manipulator. Examples of widely used controllable promoters for expression in E. coli are the lac, the trp, the tac and the lambda PL and PR promoter.

Preferably, the expression vector should also contain a terminator of transcription functional in E. coli. Examples of used terminators of transcription are the trp and the rrnB terminators.

Furthermore, the expression vector should contain a ribosome binding site, synthetic or from a natural source, allowing translation and hence expression of a downstream coding sequence. Moreover, when expression devoid of foreign sequences is desired, a unique restriction site, positioned in such a way that it allows ligation of the sequence directly to the initiation codon of the ribosome binding site, should be present.

A suitable plasmid for performing this type of expression is pKK233-2 (Pharmacia). This plasmid contains the trc promoter, the lac Z ribosome binding site and the rrnB transcription terminator.

Also suitable is plasmid pIGRI (Innogenetics, Ghent, Belgium). This plasmid contains the tetracycline resistance gene and the origin of replication of pAT₁₅₃ (available from Bioexcellence, Biores B.V., Woerden, The Netherlands), the lambda PL promoter up to the Mbo II site in the 5' untranslated region of the lambda N gene (originating from pPL(λ)); Pharmacia).

Downstream from the PL promoter, a synthetic sequence was introduced which encodes a "two cistron" translation cassette whereby the stop codon of the first cistron (being the first 25 amino acids of TNF, except for Leu at position 1 which is converted to Val) is situated between the Shine-Dalgarno sequence and the initiation codon of the second ribosome binding site. The restriction and genetic map of pIGRI is represented in Fig. 10a.

Fig. 10b and Table 5 represent respectively the nucleic acid sequence and complete restriction site analysis of pIGRI.

However, when expression as a hybrid protein is desired, then the expression vector should also contain the coding sequence of a peptide or polypeptide which is (preferably highly) expressed by this vector in the appropriate host.

In this case the expression vector should contain a unique cleavage site for one or more restriction endonucleases downstream of the coding sequence.

Plasmids pEX1, 2 and 3 (Boehringer, Mannheim) and pUEX1, 2 and 2 (Amersham) are useful for this purpose.

They contain an ampicillin resistance gene and the origin of replication of pBR322 (Bolivar et al. (1977) Gene 2, 95-113), the lac Z gene fused at its 5' end to the lambda PR promoter together with the coding sequence for the 9 first amino acids of its natural gene cro, and a multiple cloning site at the 3' end of the lac Z coding sequence allowing production of a beta galactosidase fused polypeptide.

The pUEX vectors also contain the C1857 allele of the bacteriophage lambda CI repressor gene.

Also useful is plasmid pmTNF MPH (Innogenetics). It contains the tetracycline resistance gene and the origin of replication of pAT₁₅₃ (obtainable from Bioexcellence, Biores B.V., Woerden, The Netherlands), the lambda PL promoter up to the Mbo II site in the N gene 5' untranslated region (originating from pPL(λ): Pharmacia), followed by a synthetic ribosome binding site (see sequence data), and the information encoding the first 25 AA of mTNF (except for the initial Leu which is converted to Val). This sequence is, in turn, followed by a synthetic polylinker sequence which encodes six consecutive histidines followed by several proteolytic sites (a formic acid, CNBr, kallikrein, and E. coli protease VII sensitive site, respectively), each accessible via a different restriction enzyme which is unique for the plasmid (Sma I, Nco I, Bsp MII and Stu I, respectively; see restriction and genetic map, Fig. 11a). Downstream from the polylinker, several transcription terminators are present including the E. coli trp terminator (synthetic) and the rrnBT₂ - (originating from pKK223-3; Pharmacia). The total nucleic acid sequence of this plasmid is represented in Fig. 11b.

Table 6 gives a complete restriction site analysis of pmTNF MPH.

The presence of 6 successive histidines allows purification of the fusion protein by Immobilized Metal Ion Affinity Chromatography (IMAC).

After purification, the foreign part of the hybrid protein can be removed by a suitable protein cleavage method and the cleaved product can then be separated from the uncleaved molecules using the same IMAC based purification procedure.

In all the above-mentioned plasmids where the lambda PL or PR promoter is used, the promoter is

temperature-control led by means of the expression of the lambda cl ts 857 allele which is either present on a defective prophage incorporated in the chromosome of the host (K12ΔH, ATCC n° 33767) or on a second compatible plasmid (pACYC derivative). Only in the pUEX vectors is this cl allele present on the vector itself.

5 It is to be understood that the plasmids presented above are exemplary and other plasmids or types of expression vectors maybe employed without departing from the spirit or scope of the present invention.

If a bacteriophage or phagemid is used, instead of plasmid, it should have substantially the same characteristics used to select a plasmid as described above.

10

EXAMPLE IV : SUBCLONING OF THE P₃₂ ANTIGEN IN PLASMID pIGRI :

Fifteen μg of plasmid "BS-BK-P₃₂ complet" (see Example II) was digested with Ecl XI and Bst EII (Boehringer, Mannheim) according to the conditions recommended by the supplier except that at least 3
15 units of enzyme were used per μg of DNA. Ecl XI cuts at position 226 (Fig. 5) and Bst EII at position 1136, thus approaching very closely the start and stop codon of the mature P₃₂ antigen. This DNA is hereafter called DNA coding for the "P₃₂ antigen fragment".

The DNA coding for the "P₃₂ antigen fragment" (as defined above) is subcloned in pIGRI (see fig. 10a) for expression of a polypeptide devoid of any foreign sequences. To bring the ATG codon of the expression
20 vector in frame with the P₃₂ reading frame, an intermediary construct is made in pIG2 (for restriction and genetic map, see fig. 12a; DNA sequences, see fig. 12b; complete restriction site analysis, see Table 7).

Five μg of plasmid pIG2 is digested with Nco I. Its 5' sticky ends are filled in prior to dephosphorylation.

Therefore, the DNA was incubated in 40 μl NB buffer (0.05 M Tris-Cl pH 7.4; 10 mM MgCl₂; 0.05% β-mercaptoethanol) containing 0.5 mM of all four dXTP (X = A,T,C,G) and 2 μl of Klenow fragment of E. coli
25 DNA polymerase I (5 U/μl, Boehringer, Mannheim) for at least 3 h at 15° C.

After blunting, the DNA was once extracted with one volume of phenol equilibrated against 200 mM Tris- Cl pH 8, twice with at least two volumes of diethylether and finally collected using the "gene clean kit^{T.M.}" (Bio101) as recommended by the supplier. The DNA was then dephosphorylated at the 5' ends in
30 30 μl of CIP buffer (50 mM TrisCl pH 8, 1 mM ZnCl₂) and 20 to 25 units of calf intestine phosphatase (high concentration, Boehringer, Mannheim). The mixture was incubated at 37° C for 30 min, then EGTA (ethyleneglycol bis (β-aminoethylether)-N,N',N',N' tetraacetic acid) pH 8 is added to a final concentration of 10 mM. The mixture was then extracted with phenol followed by diethylether as described above, and the DNA was precipitated by addition of 1/10 volume of 3 M KAc (Ac = CH₃COO) pH 4.8 and 2 volumes of
35 ethanol followed by storage at -20° C for at least one hour.

After centrifugation at 13000 rpm in a Biofuge A (Hereaus) for 5 min the pelleted DNA was dissolved in H₂O to a final concentration of 0.2 μg/μl.

The Ecl XI-Bst EII fragment, coding for the "P₃₂ antigen fragment" (see above) was electrophoresed on a 1% agarose gel (BRL) to separate it from the rest of the plasmid and was isolated from the gel by
40 centrifugation over a Millipore HVLP filter (Ø 2cm) (2 min., 13000 rpm, Biofuge at room temperature) and extracted with Tris equilibrated phenol followed by diethylether as described above.

The DNA was subsequently collected using the "Gene clean kit^{T.M.}" (Bio101) as recommended by the supplier.

After that, the 5' sticky ends were blunted by treatment with the Klenow fragment of E. coli DNA
45 polymerase I as described above and the DNA was then again collected using the "Gene clean kit^{T.M.}" in order to dissolve it in 7 μl of H₂O.

One μl of vector DNA is added together with one μl of 10 x ligase buffer (0.5 M TrisCl pH 7.4, 100 mM MgCl₂, 5 mM ATP, 50 mM DTT (dithiothreitol)) and 1 μl of T4 DNA ligase (1 unit/μl, Boehringer, Mannheim). Ligation was performed for 6 h at 13° C and 5 μl of the mixture is then used to transform strain
50 DH1 (lambda) [strain DH1 - ATCC N° 33849 - lysogenized with wild type bacteriophage λ] using standard transformation techniques as described for instance by Maniatis et al. in "Molecular cloning, a laboratory manual", Cold Spring Harbor Laboratory (1982).

Individual transformants are grown and lysed for plasmid DNA preparation using standard procedures (Experiments with gene fusions, Cold Spring Harbor Laboratory (1984) (T.J. Silhavy, H.L. Berman and L.W. Enquist, eds) and the DNA preparations are checked for the correct orientation of the gene within the
55 plasmid by restriction enzyme analysis.

A check for correct blunting is done by verifying the restoration of the Nco I site at the 5' and 3' end of the antigen coding sequence. One of the clones containing the P₃₂ antigen fragment in the correct

orientation is kept for further work and designated pIG₂-Mt32. In this intermediary construct, the DNA encoding the antigen is not in frame with the ATG codon. However, it can now be moved as a NcoI fragment to another expression vector.

15 15 µg of pIG₂-Mt32 is digested with Nco I. The Nco I fragment encoding the P₃₂ antigen is gel purified and blunted as described above. After purification, using "gene clear kit TM" it is dissolved in 7 µl of H₂O.

5 5 µg of plasmid pIGRI is digested with NcoI, blunted and dephosphorylated as described above. After phenol extraction, followed by diethylether and ethanolprecipitation, the pellet is dissolved in H₂O to a final concentration of 0.2 µg/µl.

10 Ligation of vector and "antigen fragment" DNA is carried out as described above. The ligation mixture is then transformed into strain DH1 (lambda) and individual transformants are analysed for the correct orientation of the gene within the plasmid by restriction enzyme analysis. A check for correct blunting is done by verifying the creation of a new Nsi I site at the 5' and 3' ends of the antigen coding sequence. One of the clones containing the P₃₂ antigen fragment in the correct orientation is kept for further work and designated pIGRI.Mt32.

15

EXAMPLE V: SUBCLONING OF THE P32 ANTIGEN IN pmTNF MPH:

20 Fifteen µg of the plasmid pIG2 Mt32 (see example IV) was digested with the restriction enzyme Nco I (Boehringer, Mannheim), according to the conditions recommended by the supplier except that at least 3 units of enzyme were used per µg of DNA.

After digestion, the reaction mixture is extracted with phenol equilibrated against 200mM TrisCl pH 8, (one volume), twice with diethylether (2 volumes) and precipitated by addition of 1:10 volume of 3 M KAc (Ac = CH₃COO) pH 4.8 and 2 volumes of ethanol followed by storage at -20 °C for at least one hour.

25 After centrifugation for 5 minutes at 13000 rpm in a Biofuge A (Hereaus) the DNA is electrophoresed on a 1% agarose gel (BRL).

30 The DNA coding for the "P₃₂ antigen fragment" as described above, is isolated by centrifugation over a Millipore HVLP filter (Ø 2cm) (2 minutes, 1300 rpm, Biofuge at room temperature) and extracted one with trisCl equilibrated phenol and twice with diethylether. The DNA is subsequently collected using "Gene clean kit T.M." (Bio 101) and dissolved in 7µl of H₂O.

35 The 5' overhanging ends of the DNA fragment generated by digestion with Nco I were filled in by incubating the DNA in 40 µl NB buffer (0.05 M Tris-HCl, pH 7.4; 10 mM MgCl₂; 0.05% β-mercaptoethanol) containing 0.5 mM of all four dXTPS (X = A, T, C, G) and 2µl of Klenow fragment of E. coli DNA polymerase I (5 units/µl Boehringer Mannheim) for at least 3 h at 15 °C. After blunting, the DNA was extracted with phenol, followed by diethylether, and collected using a "gene clean kit T.M." as described above.

Five µg of plasmid pmTNF MPH is digested with Stu I, subsequently extracted with phenol, followed by diethylether, and precipitated as described above. The restriction digest is verified by electrophoresis of a 0.5 µg sample on an analytical 1.2% agarose gel.

40 The plasmid DNA is then desphosphorylated at the 5' ends to prevent self-ligation in 30µl of CIP buffer (50 mM TrisCl pH 8, 1mM ZnCl₂) and 20 to 25 units of calf intestine phosphatase (high concentration, Boehringer Mannheim). The mixture is incubated at 37 °C for 30 minutes, then EGTA (ethyleneglycol bis (β-aminoethylether)-N,N,N',N' tetraacetic acid) pH8 is added to a final concentration of 10mM. The mixture is extracted with phenol followed by diethylether and the DNA is precipitated as described above. The precipitate is pelleted by centrifugation in a Biofuge A (Hereaus) at 13000 rpm for 10 min at 4 °C and the pellet is dissolved in H₂O to a final DNA concentration of 0.2 µg/µl.

45 One µl of this vector DNA is mixed with the 7 µl solution containing the DNA fragment coding for the "P32antigen fragment" (as defined above) and 1 µl 10 x ligase buffer (0.5 M TrisCl pH7.4, 100 mM MgCl₂, 5 mM ATP, 50 mM DTT (dithiothreitol)) plus 1 µl T₄ DNA ligase (1 unit/µl, Boehringer Mannheim) is added. The mixture is incubated at 13 °C for 6 hours and 5 µl of the mixture is then used for transformation into strain DH1(lambda, using standard transformation techniques are described by for instance Maniatis et al. in "Molecular cloning, a laboratory manual", Cold Spring Harbor Laboratory (1982).

50 Individual transformants are grown and then lysed for plasmid DNA preparation using standard procedures (Experiments with gene fusions, Cold Spring Harbor Laboratory 1984 (T.J. Silhavy, M.L. Berman and L.W. Enquist eds.)) and are checked for the correct orientation of the gene within the plasmid by restriction enzyme analysis.

One of the clones containing the DNA sequence encoding the antigen fragment in the correct orientation was retained for further work and designated pmTNF-MPH-Mt32. It encodes all information of the

P₃₂ antigen starting from position +4 in the amino acid sequence (see fig. 5). The amino acid sequence of the total fusion protein is represented in fig. 13.

5 EXAMPLE VI: INDUCTION OF ANTIGEN EXPRESSION FROM pmTNF MPH Mt32 :

A- MATERIAL AND METHODS

10 DNA of pmTNF-MPH-Mt32 is transformed into *E. coli* strain K12ΔH (ATCC 33767) using standard transformation procedures except that the growth temperature of the cultures is reduced to 28° C and the heat shock temperature to 34° C.

A culture of K12ΔH harboring pmTNF-MPH-Mt32, grown overnight in Luria broth at 28° C with vigorous shaking in the presence of 10 μg/ml tetracycline, is inoculated into fresh Luria broth containing tetracycline (10 μg/ml) and grown to an optical density at 600 nanometers of 0.2 in the same conditions as for the
15 overnight culture.

When the optical density at 600 nanometers has reached 0.2 half of the culture is shifted to 42° C to induce expression while the other half remains at 28° C as a control. At several time intervals aliquots are taken which are extracted with one volume of phenol equilibrated against M9 salts (0.1% ammonium chloride, 0.3% potassium dihydrogenium phosphate, 1.5% disodium hydrogenium phosphate, 12 molecules of water) and 1% SDS. At the same time, the optical density (600 nm) of the culture is checked. The proteins are precipitated from the phenol phase by addition of two volumes of acetone and storage overnight at -20° C. The precipitate is pelleted (Biofuge A, 5 min., 13000 rpm, room temperature) dried at the air, dissolved in a volume of Laemmli (Nature (1970) 227 :680) sample buffer (+ β mercapto ethanol)
25 according to the optical density and boiled for 3 min.

Samples are then run on a SDS polyacrylamide gel (15%) according to Laemmli (1970). Temperature induction of mTNF-His₆-P₃₂ is monitored by both Coomassie Brilliant Blue (CBB) staining and immunoblotting. CBB staining is performed by immersing the gel in a 1/10 diluted CBB staining solution (0.5 g CBB-R250 (Serva) in 90 ml methanol : H₂O (1:1 v/v) and 10 ml glacial acetic acid) and left for about one hour on
30 a gently rotating platform. After destaining for a few hours in destaining solution (30% methanol, 7% glacial acetic acid) protein bands are visualised and can be scanned with a densitometer (Ultrosan XL Enhanced Laser Densitometer, LKB).

For immunoblotting the proteins are blotted onto Hybond C membranes (Amersham) as described by Townbin et al (1979). After blotting, proteins on the membrane are temporarily visualised with Ponceau S (Serva) and the position of the molecular weight markers is indicated. The stain is then removed by washing in H₂O. Aspecific protein binding sites are blocked by incubating the blots in 10% non-fat dried milk for about 1 hour on a gently rotating platform. After washing twice with NT buffer (25 mM Tris-HCl, pH 8.0; 150 mM NaCl) blots are incubated with polyclonal rabbit anti-32-kDa antiserum (1:1000), obtained as described in example I ("screening of the λgt11 M. tuberculosis recombinant DNA library with anti-32-kDa antiserum")
40 in the presence of *E. coli* lysate or with monoclonal anti-hTNF-antibody which crossreacts with mTNF (Innogenetics, n° 17F5D10) for at least 2 hours on a rotating platform. After washing twice with NT buffer + 0.02% Triton.X.100, blots are incubated for at least 1. hour with the secondary antiserum : alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (1/500; Prosan) in the first case, and alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins (1/500; Sigma) in the second case.

45 Blots are washed again twice with NT buffer + 0.02% Triton X100 and visualisation is then performed with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) from Promega using conditions recommended by the supplier.

50 B. RESULTS

Upon induction of K12ΔH cells containing pmTNF-MPH-Mt32, a clearly visible band of about 35-kDa appears on CBB stained gels, already one hour after start of induction (Fig. 14a). This band, corresponding to roughly 25% of total protein contents of the cell, reacts strongly with anti-32-kDa and anti-mTNF antisera
55 on immunoblots (Fig. 14b). However, this band represents a cleavage product of the original fusion protein, since a minor band, around 37 kDa, is also visible on immunoblots, reacting specifically with both antisera as well. This suggests that extensive cleavage of the recombinant mTNF-His₆-P₃₂ takes place about 2-3 kDa from its carboxyterminal end.

EXAMPLE VII : PURIFICATION OF RECOMBINANT ANTIGEN ON IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (IMAC) :

The hybrid protein mTNF-His₆-P₃₂ (amino acid sequence, see fig. 13) expressed by K12ΔH cells containing pmTNF.MPH.Mt32, is especially designed to facilitate purification by IMAC, since the 6 successive histidines in the polylinker sequence bring about a strong affinity for metal ions (HOCHULI et al, 1988).

10 a. Preparation of the crude cell extract :

12 l of E. coli cells K12ΔH containing plasmid pmTNF-MPH-Mt32 were grown in Luria Broth containing tetracycline (10 μg/ml) at 28° C to an optical density (600 nm) of 0.2 and then induced by shifting the temperature to 42° C. After 3 hours of induction, cells were harvested by centrifugation (Beckman, JA 10 rotor, 7.500 rpm, 10 min). The cell paste was resuspended in lysis buffer (10 mM KCl, 10 mM Tris-HCl pH 6.8, 5 mM EDTA) to a final concentration of 50% (w/v) cells.

ε-NH₂-capronic acid and dithiotreitol (DTT) were added to a final concentration of resp. 20 mM and 1 mM, to prevent proteolytic degradation. This concentrated cell suspension was stored overnight at -70° C.

Cells were lysed by passing them three times through a French press (SLM-Aminco) at a working pressure of 800-1000 psi. During and after lysis, cells were kept systematically on ice.

The cell lysate was cleared by centrifugation (Beckman, JA 20, 18.000 rpm, 20 min, 4° C). The supernatant (SN) was carefully taken off and the pellet, containing membranes and inclusion bodies, was kept for further work since preliminary experiments had shown that the protein was mainly localised in the membrane fraction.

7 M guanidinium hydrochloride (GuHCl, marketed by ICN) in 100 mM phosphate buffer pH 7.2 was added to the pellet volume to a final concentration of 6 M GuHCl. The pellet was resuspended and extracted in a bounce tissue homogenizer (10 cycles).

After clearing (Beckman, JA 20, 18.000 rpm, 20 min, 4° C), about 100 ml of supernatant was collected (= extract 1) and the removing pellet was extracted again as described above (= extract 2, 40 ml).

The different fractions (SN, EX1, EX2) were analysed on SDS-PAGE (Laemmli, Nature 1970; 227:680) together with control samples of the induced culture. Scanning of the gel revealed that the recombinant protein makes up roughly 25% of the total protein content of the induced cell culture. After fractionation most of the protein was found back in the extracts. No difference was noticed between reducing and non-reducing conditions (plus and minus β-mercaptoethanol).

15 b. Preparation of the Ni⁺⁺ IDA (Imino diacetic acid) column :

5 ml of the chelating gel, Chelating Sepharose 6B (Pharmacia) is washed extensively with water to remove the ethanol in which it is stored and then packed in a "Econo-column" (1 x 10 cm. Biorad). The top of the column is connected with the incoming fluid (sample, buffer, etc) while the end goes to the UV₂₈₀ detector via a peristaltic pump. Fractions are collected using a fraction collector and, when appropriate, pH of the fractions is measured manually.

The column is loaded with Ni⁺⁺ (6 ml NiCl₂.6H₂O; 5 μg/μl) and equilibrated with starting buffer (6 M guanidinium hydrochloride, 100 mM phosphate buffer, pH 7.2).

After having applied the sample, the column is washed extensively with starting buffer to remove unbound material.

To elute the bound material, 2 different elution procedures are feasible :

1) elution by decreasing pH,

2) elution by increasing imidazol concentration.

Both will be discussed here.

To regenerate the column, which has to be done after every 2-3 runs, 20 ml (about 5 column volumes) of the following solutions are pumped successively through the column :

- 0.05 M EDTA - 0.5 M NaCl

- 0.1 M NaOH

- H₂O

- 6 ml NiCl₂.6H₂O (5 mg/ml).

After equilibrating with starting buffer the column is ready to use again.

c. Chromatography :

All buffers contained 6 M guanidinium hydrochloride throughout the chromatography. The column was developed at a flow rate of 0.5 ml/min at ambient temperature. Fractions of 2 ml were collected and, when appropriate, further analysed by SDS-PAGE and immunoblotting. Gels were stained with Coomassie Brilliant Blue R250 and silver stain, as described by ANSORGE (1985). Immunoblotting was carried out as described in example I. The primary antiserum used was either polyclonal anti-32kDa-antiserum (1/1000) obtained as described in example 1 ("screening of the *λ*gt11 M. tuberculosis recombinant DNA library with anti-32kDa-antiserum") or anti-*E. coli* -immunoglobulins (1/500; PROSAN), or monoclonal anti-hTNF-antibody which cross-reacts with mTNF (Innogenetics, N° 17F5D10). The secondary antiserum was alkaline phosphatase conjugated swine anti-rabbit immunoglobulins (1/500, PROSAN), or alkaline phosphatase conjugated rabbit-anti-mouse immunoglobulins (1/500, Sigma).

15 C1. Elution with decreasing pH :

Solutions used :

A : 6 M GuHCl 100 mM phosphate pH 7.2

B : 6 M GuHCl 25 mM phosphate pH 7.2

20 C : 6 M GuHCl 50 mM phosphate pH 4.2

After applying 3 ml of extract 1 ($OD_{280} = 32.0$) and extensively washing with solution A, the column is equilibrated with solution B and then developed with a linear pH gradient from 7.2 to 4.2 (25 ml of solution B and 25 ml of solution C were mixed in a gradient former). The elution profile is shown in figure 15.

From SDS-PAGE analysis (Coomassie and silverstain) it was clear that most of the originally bound 25 recombinant protein was eluted in the fractions between pH 5.3 and 4.7.

Screening of these fractions on immunoblot with anti-32-kDa and the 17F5D10 monoclonal antibody showed that, together with the intact recombinant protein, also some degradation products and higher aggregation forms of the protein were present, although in much lower amount. Blotting with anti-*E. coli* antibody revealed that these fractions (pH 5.3-4.7) still contained immunodetectable contaminating *E. coli* 30 proteins (75, 65, 43, 35 and 31 kDa bands) and lipopolysaccharides..

C2. Elution with increasing imidazol concentration :

35 Solutions used :

A : 6 M GuHCl 100 mM phosphate pH 7.2

B : 6 M GuHCl 50 mM imidazol pH 7.2

C : 6 M GuHCl 100 mM imidazol pH 7.2

D : 6 M GuHCl 15 mM imidazol pH 7.2

40 E : 6 M GuHCl 25 mM imidazol pH 7.2

F : 6 M GuHCl 35 mM imidazol pH 7.2

Sample application and washing was carried out as in C1, except that after washing, no equilibration was necessary with 6 M GuHCl 25 mM phosphate. The column was first developed with a linear gradient of imidazol going from 0 to 50 mM (25 ml of solution A and 25 ml of solution B were mixed in a gradient 45 former) followed by a step elution to 100 mM imidazol (solution C). During the linear gradient, proteins were gradually eluted in a broad smear, while the step to 100 mM gave rise to a clear peak (fig. 16).

SDS-PAGE analysis of the fractions revealed that in the first part of the linear gradient (fr 1-24) most contaminating *E. coli* proteins were washed out, while the latter part of the gradient (fr 25-50) and the 100 mM peak contained more than 90% of the recombinant protein.

50 As in C1, these fractions showed, besides a major band of intact recombinant protein, some minor bands of degradation and aggregation products. However, in this case, the region below 24-kDa seemed nearly devoid of protein bands, which suggests that less degradation products co-elute with the intact protein. Also, the same contaminating *E. coli* proteins were detected by immunoblotting, as in C1, although the 31-kDa band seems less intense and even absent in some fractions.

55 In a second stage, we developed the column with a step gradient of increasing imidazol concentrations. After having applied the sample and washed the column, 2 column volumes (about 8 ml) of the following solutions were brought successively onto the column : solution D, E, F and finally 4 column volumes of solution C. The stepgradient resulted in a more concentrated elution profile (fig. 17) which makes it more

suitable for scaling up purposes.

In conclusion, the mTNF-His₆-P₃₂ protein has been purified to at least 90% by IMAC. Further purification can be achieved through a combination of the following purification steps :

- IMAC on chelating superose (Pharmacia)
 - 5 - ion exchange chromatography (anion or cation)
 - reversed phase chromatography
 - gel filtration chromatography
 - immunoaffinity chromatography
 - elution from polyacrylamide gel.
- 10 These chromatographic methods are commonly used for protein purification.
The plasmids of figures 10b, 11b and 12b are new.

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Claims

1. Recombinant polypeptide containing in its polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity

- constituted by amino acid at position (31) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 3a and fig. 3b, or
 - the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 3a and fig. 3b, or
 - the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 3a and fig. 3b, or
 - the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 3a and fig. 3b, or
 - the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 3a and fig. 3b, or
 - the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :
- the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or
- react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,
- and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.
2. Recombinant polypeptide according to claim 1, containing in its polypeptidic chain, one at least of the following amino acid sequences:
- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b, or
 - the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 4a and fig. 4b, or
 - the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 4a and fig. 4b, or
 - the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 4a and fig. 4b, or
 - the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 4a and fig. 4b, or
 - the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 4a and fig. 4b, or
 - the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 4a and fig. 4b, or
 - the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :
- the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or
- react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,
- and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.
3. Recombinant polypeptide according to claim 1, containing in its polypeptidic chain, one at least of the following amino acid sequences:
- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (-1) represented on fig. 5, or
 - the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 5, or
 - the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 5, or
 - the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 5, or
 - the one extending from the extremity constituted by amino acid at position (101) to the extremity

constituted by amino acid at position (120) represented on fig. 5, or

- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 5, or

- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 5, or

5 - the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (295) represented on fig. 5,

and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :

10 the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or

react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,

15 and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (295) represented on fig. 5.

4. Recombinant polypeptide according to claim 1, containing in its polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

20 - the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,

25 - the one extending from the extremity constituted by amino acid at position (-49) to to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,

30 - the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

35 - the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

40 - the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

5. Recombinant polypeptide according to claim 2, containing in its polypeptidic chain, one at least of the following amino acid sequences:

45 - the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

50 - the one extending from the extremity constituted by amino acid at position (-49) to to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

55 - the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity

- [illegible]

- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 5,
- 5 - the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (295) represented on fig. 5.
- 10 10. Amino acid sequences constituted by a polypeptide according to claims 1 to 9, and a protein or an heterologous sequence with respect to said polypeptide, said protein or heterologous sequence comprising from about 1 to about 1000 amino acids.
- 11. Amino acid sequence according to claim 10, wherein the heterologous protein is β -galactosidase.
- 15 12. Nucleic acid comprising
 - a nucleotide sequence coding for anyone of the polypeptides according to claims 1 to 11,
 - or nucleotide sequences which hybridize with the nucleotide sequences coding for anyone of the polypeptides according to claims 1 to 11,
 - or nucleotide sequences which are complementary to the nucleotide sequences coding for any of the
- 20 polypeptides according to claims 1 to 11,
- the above mentioned nucleotide sequences wherein T can be replaced by U.
- 13. Nucleic acid according to claim 12, comprising one at least of the following nucleotide sequences:
 - the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 3a and fig. 3b,
 - 25 - the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
 - the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
 - the one extending from the extremity constituted by nucleotide at position (1242) to the extremity
- 30 constituted by nucleotide at position (1358), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 3a and fig. 3b,
- or above said nucleotide sequences wherein T is replaced by U,
- or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.
- 35 14. Nucleic acid according to claim 13, comprising one at least of the following nucleotide sequences:
 - the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 4a and fig. 4b,
 - the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
 - 40 - the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
 - the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 4a and fig. 4b,
 - 45 or above said nucleotide sequences wherein T is replaced by U,
 - or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.
 - 15. Nucleic acid according to claim 13, comprising one at least of the following nucleotide sequences:
 - the one extending from the extremity constituted by nucleotide at position (130) to the extremity
 - 50 constituted by nucleotide at position (219) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (1104) to the extremity constituted by nucleotide at position (1299),
 - 55 or above said nucleotide sequences wherein T is replaced by U,
 - or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.
 - 16. Nucleic acid according to claim 13, comprising one of the following sequences:

- [illegible]

- [illegible]

- [illegible]

- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
 - the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
 - 5 - the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
 - the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b.
21. Nucleic acid according to claim 15, consisting in one of the following nucleotide sequences:
- 10 - the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (129) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted
 - 15 by nucleotide at position (1104) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
 - 20 - the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (130) to the extremity
 - 25 constituted by nucleotide at position (219) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
 - 30 - the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
 - the one extending from the extremity constituted by nucleotide at position (1104) to the extremity
 - 35 constituted by nucleotide at position (1299) represented in fig. 5.
22. Recombinant nucleic acid containing at least one of the nucleotide sequences according to claims 13 to 21, inserted in a heterologous nucleic acid.
23. DNA or RHA primer constituted by one of the following sequences:
- A (i) CAGCTTGTGACAGGGTTCGTGGC
 - 40 A (ii) GGTTCGTGGCGCCGTCACG
 - A (iii) CGTCGCGCGCCTAGTGTCGG
 - A (iv) CGGCGCCGGTCGGTGGCACGGCGA
 - A (v) CGTCGGCGCGGCCCTAGTGTCGG
 - B TCGCCCGCCCTGTACCTG
 - 45 C GCGCTGACGCTGGCGATCTATC
 - D CCGCTGTTGAACGTCGGGAAG
 - E AAGCCGTCGGATCTGGGTGGCAAC
 - F (i) ACGGCACTGGGTGCCACGCCCAAC
 - F(ii) ACGCCCAACACCGGGCCCCGCCGCA
 - 50 F (iii) ACGGGCACTGGGTGCCACGCCCAAC
 - F (iv) ACGCCCAACACCGGGCCCCGCCGCCA
24. DNA or RNA primer set constituted by any of the nucleotide sequences A(i), A(ii), A(iii), A(iv), A(v), B, C, D, E, F(i), F(ii), F(iii) or F(iv) in association with the complement of any other nucleotide sequences chosen from A, B, C, D, E, or F, A meaning any of the sequences A(i), A(ii), A(iii), A(iv), A(v) and F any of the
- 55 sequences F(i), F(ii), F(iii) and F(iv),
A(i), A(ii), A(iii), A(iv), A(v), B, C, D, E, F(i), F(ii), F(iii) and F(iv) having the meaning of claim 11, and advantageously constituted by the following elements :

A(i)

or A(ii)

or A(iii)

or A(iv)

or A(v)

and the complement of B

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	A(i)	
	or A(ii)	
5	or A(iii)	and the complement of C
	or A(iv)	
	or A(v)	
10	B	and the complement of C
	A(i)	
15	or A(ii)	
	or A(iii)	and the complement of F
	or A(iv)	
20	or A(v)	
	A(i)	
	or A(ii)	
25	or A(iii)	and the complement of D
	or A(iv)	
	or A(v)	
30	A(i)	
	or A(ii)	
	or A(iii)	and the complement of E
35	or A(iv)	
	or A(v)	
40	B	and the complement of D
	B	and the complement of E
	B	and the complement of F
	C	and the complement of D
45	C	and the complement of E
	C	and the complement of F
	D	and the complement of E
50	D	and the complement of F
	E	and the complement of F.

25. Recombinant vector, particularly for cloning and/or expression, comprising a vector sequence, notably of the type plasmid, cosmid or phage, and a recombinant nucleic acid according to anyone of claims 13 to 21, in one of the non essential sites for its replication.

26. Recombinant vector according to claim 25, containing in one of its non essential sites for its replication necessary elements to promote the expression of polypeptides according to anyone of claims 1 to 12 in a

cellular host and possibly a promoter recognized by the polymerase of the cellular host, particularly an inducible promoter and possibly a signal sequence and/or an anchoring sequence.

27. Recombinant vector according to claim 26, containing the elements enabling the expression by *E. coli* of a nucleic acid according to anyone of claims 6 to 9 inserted in the vector, and particularly the elements enabling the expression of the gene or part thereof of β -galactosidase.

28. Cellular host which is transformed by a recombinant vector according to anyone of claims 25 to 27, and comprising the regulation elements enabling the expression of the nucleotide sequence coding for the polypeptide according to anyone of claims 1 to 12 in this host.

29. Cellular host according to claim 28, chosen from among bacteria such as *E. coli*, transformed by the vector according to claim 25, or chosen from among eukaryotic organism, transformed by the vector according to claim 25.

30. Expression product of a nucleic acid expressed by a transformed cellular host according to anyone of claims 28 or 29.

31. Antibody characterized by the fact that it is directed against a recombinant polypeptide according to anyone of claims 1 to 12.

32. Nucleotidic probes, hybridizing with anyone of the nucleic acids according to claims 13 to 21 or with their complementary sequences, and particularly the probes chosen among the following nucleotidic sequences

Probes A(i) A(ii), A(iii) and A(iv)

A (i) CAGCTTGTGACAGGGTTTCGTGGC

A(ii) GGTTCGTGGCGCCGTCACG

A (iii) CGTCGCGCGCCTAGTGTCCG

A (iv) CGGCGCCGTCGGTGGCAGGCGA

A (v) CGTCGGCGCGGCCCTAGTGTCCG

Probe B

TCGCCCGCCCTGTACCTG

Probe C

GCGCTGACGCTGGCGATCTATC

Probe D

CCGCTGTTGAACGTCGGGAAG

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

Probes F(i) and F(ii)

F (i) ACGGCACTGGGTGCCACGCCCAAC

F (ii) ACGCCCAACACCGGGCCCGCCGCA

F (iii) ACGGGCACTGGGTGCCACGCCCAAC

F (iv) ACGCCCAACACCGGGCCCGCGCCCA

or their complementary nucleotidic sequences.

33. Process for preparing a recombinant polypeptide according to anyone of claims 1 to 12 comprising the following steps:

- the culture in an appropriate medium of a cellular host which has previously been transformed by an appropriate vector containing a nucleic acid according to anyone of claims 12 to 22, and
- the recovery of the polypeptide produced by the above said transformed cellular host from the above said culture medium.

34. Method for the *in vitro* diagnostic of tuberculosis in a patient liable to be infected by *Mycobacterium tuberculosis* comprising the following steps:

- the possible previous amplification of the amount of the nucleotide sequences according to anyone of claims 12 to 22, liable to be contained in a biological sample taken from said patient by means of a DNA primer set according to claim 24,
- contacting the above mentioned biological sample with a nucleotide probe according to claim 32, under conditions enabling the production of an hybridization complex formed between said probe and said nucleotide sequence,
- detecting the above said hybridization complex which has been possibly formed.

35. Method for the *in vitro* diagnostic of tuberculosis in a patient liable to be infected by *Mycobacterium tuberculosis* comprising

- contacting a biological sample taken from a patient with a polypeptide according to anyone of claims 1 to 11, under conditions enabling an *in vitro* immunological reaction between said polypeptide and the antibodies which are possibly present in the biological sample and

- the in vitro detection of the antigen/antibody complex which has been possibly formed.

36. Method for the in vitro diagnostic of tuberculosis in a patient liable to be infected by M. tuberculosis, comprising the following steps:

- contacting the biological sample with an appropriate antibody according to claim 31, under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. tuberculosis which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

37. Necessary or kit for an in vitro diagnostic method of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis according to claim 34, comprising

- a determined amount of a nucleotide probe according to claim 32,
- advantageously the appropriate medium for creating an hybridization reaction between the sequence to be detected and the above mentioned probe,
- advantageously, reagents enabling the detection of the hybridization complex which has been formed between the nucleotide sequence and the probe during the hybridization reaction.

38. Necessary or kit for an in vitro diagnostic method of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis according to claim 35, comprising

- a polypeptide according to anyone of claims 1 to 11,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complex which has been produced by the immunological reaction, said reagents possibly having a label, or being liable to be recognized by a labeled reagent, more particularly in the case where the above mentioned polypeptide is not labeled.

39. Necessary or kit for an in vitro diagnostic method of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis according to claim 36, comprising

- an antibody according to claim 31,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complexes which have been produced by the immunological reaction, said reagents possibly having a label or being liable to be recognized by a label reagent, more particularly in the case where the above mentioned antibody is not labeled.

40. Immunogenic composition comprising a polypeptide according to anyone of claims 1 to 11, in association with a pharmaceutically acceptable vehicle.

41. Vaccine composition comprising among other immunogenic principles anyone of the polypeptides according to claims 1 to 11 or the expression product of claim 30, possibly coupled to a natural protein or to a synthetic polypeptide having a sufficient molecular weight so that the conjugate is able to induce in vivo the production of antibodies neutralizing Mycobacterium tuberculosis, or induce in vivo a cellular immune response by activating M. tuberculosis antigen-responsive T cells.

42. Process for the enzymatical amplification of a nucleotide seance according to claims 12 to 22, and detection of the amplified nucleotide sequence, wherein

- the amplification is achieved by PCR technique by means of a primer set and the detection of the PCR amplified product is achieved by a hybridization reaction with a detection probe constituted by an oligonucleotide sequence of at least 10 nucleotides, said sequence being located between the two PCR primers which have been used for amplifying said nucleotide sequence,
- the primer set and detection probe used being preferably chosen among the following elements:

Primer set

P1 GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG

P2 compl. GTACCACTCGAACGCCGGGGTGTGAT

Probe B

TCGCCCGCCCTGTACCTG

Primer set

P1 GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG

P3 compl. TCCCACTTGTAAGTCTGGCA

Probe B

TCGCCCGCCCTGTACCTG

Primer set

P1 GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG

P4 compl. CGGCAGCTCGCTGGTCAGGA

Probe B

TCGCCCGCCCTGTACCTG

Primer set

- P1 GAGTACCTGCAGGTGCCGTGCGCGTCGATGGGCGG
P5 compl. GCGTCACCCATCGCCAGGCCGATCAGG
Probe B
TCGCCCGCCCTGTACCTG or
- 5 Probe C
GCGCTGACGCTGGCGATCTATC
Primer set
P1 GAGTACCTGCAGGTGCCGTGCGCGTCGATGGGCGG
P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT
- 10 Probe B
TCGCCCGCCCTGTACCTG or
Probe C
GCGCTGACGCTGGCGATCTATC or
Probe D
CCGCTGTTGAACGTCGGGAAG or
- 15 Probe E
AAGCCGTCGGATCTGGGTGGCAAC
Primer set
P2 ATCAACACCCCGGCGTTTCGAGTGGTAC
P5 compl. GCGTCACCCATCGCCAGGCCGATCAGG
- 20 Probe C
GCGCTGACGCTGGCGATCTATC
Primer set
P2 ATCAACACCCCGGCGTTTCGAGTGGTAC
P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT
- 25 Probe C
GCGCTGACGCTGGCGATCTATC or
Probe D
CCGCTGTTGAACGTCGGGAAG or
- 30 Probe E
AAGCCGTCGGATCTGGGTGGCAAC
Primer set
P3 TGCCAGACTTACAAGTGGGA
P5 compl. GCGTCACCCATCGCCAGGCCGATCAGG
- 35 Probe C
GCGCTGACGCTGGCGATCTATC
Primer set
P3 TGCCAGACTTACAAGTGGGA
P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT
- 40 Probe C
GCGCTGACGCTGGCGATCTATC or
Probe D
CCGCTGTTGAACGTCGGGAAG or
Probe E
AAGCCGTCGGATCTGGGTGGCAAC
- 45 Primer set
P4 TCCTGACCAGCGAGCTGCCG
P5 compl. GCGTCACCCATCGCCAGGCCGATCAGG
- 50 Probe C
GCGCTGACGCTGGCGATCTATC
Primer set
P4 TCCTGACCAGCGAGCTGCCG
P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT
- 55 Probe C
GCGCTGACGCTGGCGATCTATC or
Probe D
CCGCTGTTGAACGTCGGGAAG or
Probe E

AAGCCGTCGGATCTGGGTGGCAAC

Primer set

P5 CCTGATCGGCCTGGCGATGGGTGACGC

P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT

5 Probe D

CCGCTGTTGAACGTCGGGAAG or

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

10 or the primer set being preferably chosen among the primer sets according to claim 24, and the detection probe being constituted by any oligonucleotide sequence of at least 10 nucleotides, said sequence being located between the two PCR primers constituting the primer set which has been used for amplifying said nucleotide sequence.

43. A vector sequence forming part of a recombinant vector according to claim 25, said vector sequence having either the nucleic acid sequence represented in fig. 10b, or the nucleic acid sequence represented in fig. 11b, or the nucleic acid sequence represented in fig. 12b.

44. Plasmids comprising either the nucleic acid sequence of fig. 10b, or the nucleic acid sequence of fig. 11b, or the nucleic acid sequence of fig. 12b.

45. Peptides of claim 1, advantageously used to produce antibodies, particularly monoclonal antibodies and which have the following amino acid sequences :

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25	Amino acid position (NH ₂ -terminal)		Amino acid position (COOH-terminal)
	12	QVPSPSMGRDIKVQFQSGGA	31
	36	LYLLDGLRAQDDFSGWDINT	55
30	77	SFYSDWYQPACRKAGCQTYK	96
	101	LTSELPGWLQANRHVKPTGS	120
	175	KASDMWGPKEPAWQRNDPL	194
35	211	CGNGKPSDLGGNNLPAKFLE	230
	275	KPDLQRHWVPRPTGPPQGA	294
	77	SFYSDWYQPACGKAGCQTYK	96
40	276	PDLQRALGATPNTGPAPQGA	299

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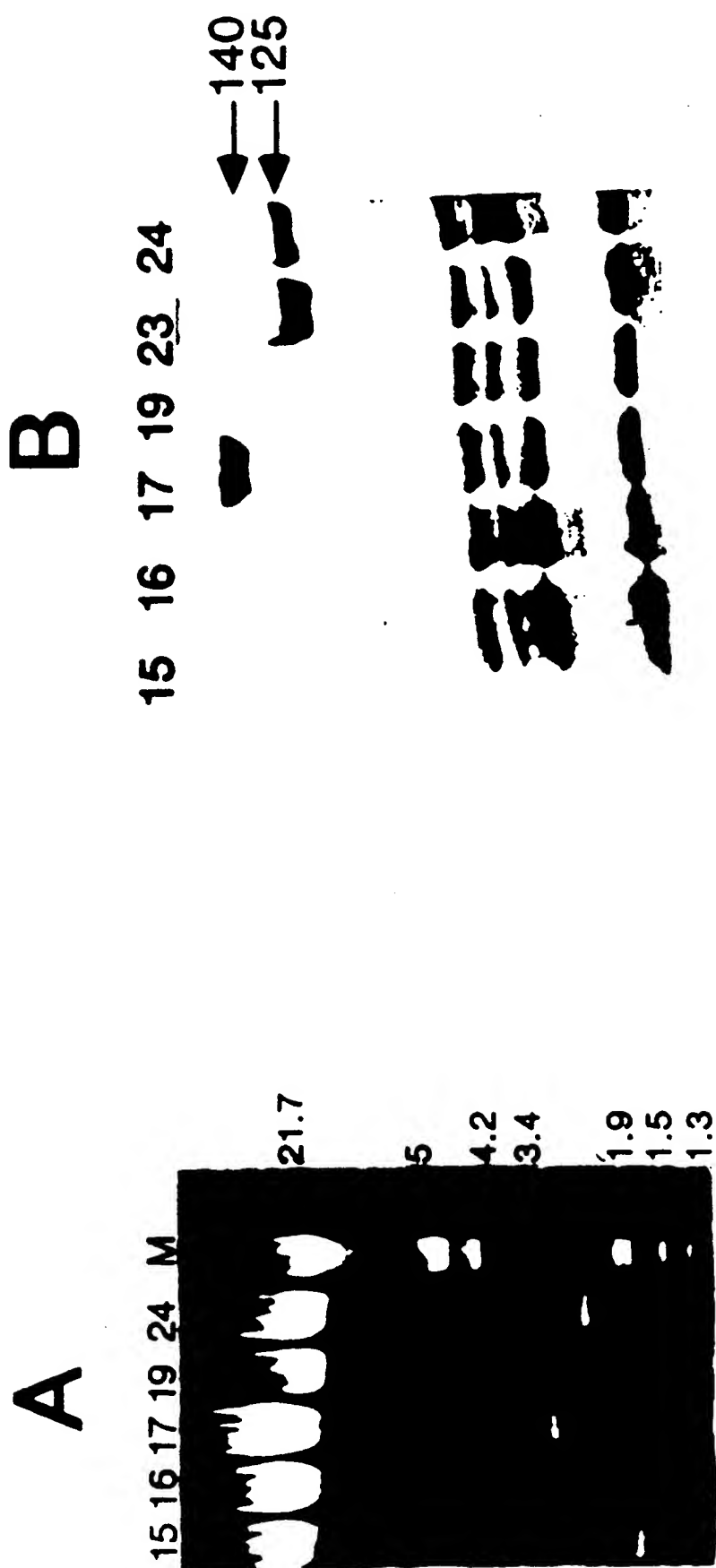
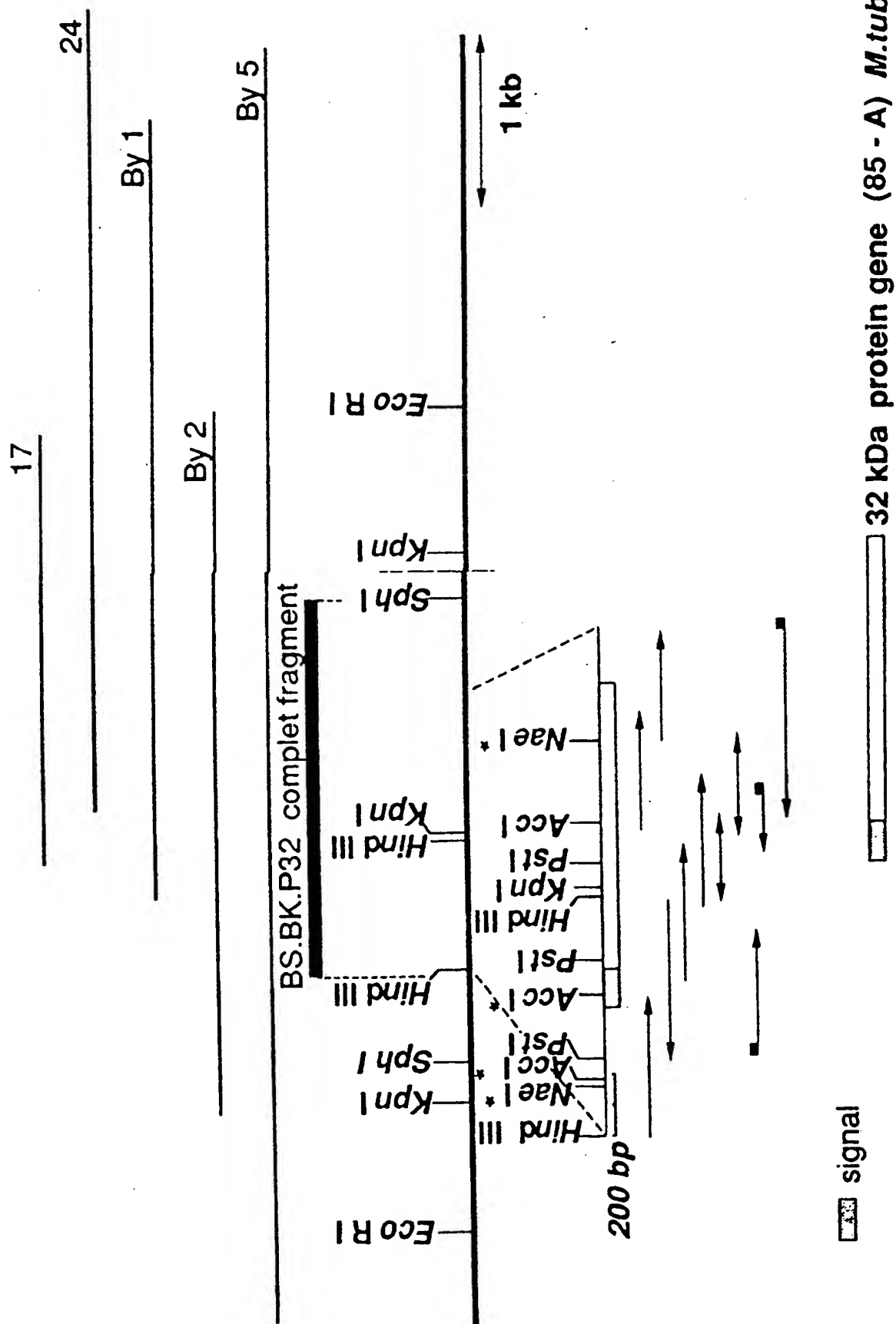


Figure 1

Figure 2



32 kDa protein gene (85 - A) *M. tuberculosis*

CGACACATGCCCCAGACACTGCGGAAATGCCACCTTCAGGCCGTCCGGTCGGT
 CCGGAA **TTGGC** CGTGAAACGACCGCCGG **ATAA** GGGTTTCGGCGGTGCGCTTGATGCGGGT
 GGACGCCCCA¹⁸³AGTTGTGTTGACTACACGAGCACTGCCGGGCCAGCGCCTGCAGTCTGACCT
 AATCAGGATGCGCCCAAAC¹⁹⁵ATGCAATGGATGCG **TTGAG²³A** **TGAG²¹⁹GATG²¹⁹GGHAGCA** **AGA**
 MET-ARG-PRO-ASN-MET-HIS- GLY-CYS-VAL- GLU- MET- ARG-MET-ARG-GLU-ALA-ARG
 -59 -49 -47
 234 **ATG**-CAG-CTT-GTT-GAC-AGG-GTT-CGT-GGC-GCC-GTC-ACG-GGT-ATG-TCG-CGT-CGA-CTC-GTG-GTC-
 -42 MET-GLN-LEU-VAL-ASP-ARG-VAL-ARG-GLY-ALA-VAL-THR-GLY-MET-SER-ARG-ARG-LEU-VAL-VAL-
 -29
 294 GGG-GCC-GTC-XCG-CXC-YTA-GTG-TCG-GGT-GTC-GGC-GCC-GTC-GGT-GGC-ACG-GCG-ACC-GCG-
 -22 GLY-ALA-VAL-a₁-b₁-LEU-VAL-SER-GLY-LEU-VAL-GLY-ALA-VAL-GLY-GLY-THR-ALA-THR-ALA-
 354 GGG-GCA-TTT-TCC-CGG-CGG-TTG-CCG-GTG-GAG-TAC-CTG-CAG-GTG-CCG-TCG-CCG-TCG-ATG-
 -2 GLY-ALA-phe-ser-arg-pro-gly-leu-pro-val-glu-tyr-leu-gln-val-pro-ser-pro-ser-met-
 -1 +1
 414 GGC-CGT-GAC-ATC-AAG)-GTC-CAA-TTC-CAA-AGT-GGT-GGT-GCC-AAC-TCG-CCC-GCC-CTG-TAC-CTG-
 19 gly-arg-asp-ile-lys-val-gln-phe-gln-ser-gly-gly-ala-asn-ser-pro-ala-leu-tyr-leu-
 ↓ 17
 474 CTC-GAC-GGC-CTG-CGC-CAG-GAC-GAC-TTC-AGC-GGC-TGG-GAC-ATC-AAC-ACC-CCG-GCG-TTC-
 39 leu-asp-gly-leu-arg-ala-gln-asp-phe-ser-gly-trp-asp-ile-asn-thr-pro-ala-phe-
 534 GAG-TGG-TAC-GAC-CAG-TCG-GGC-CTG-TCG-GTG-GTC-ATG-CCG-GTG-GGT-GGC-CAG-TCA-AGC-TTC-
 59 glu-trp-tyr-asp-gln-ser-gly-leu-ser-val-met-pro-val-gly-gly-gln-ser-ser-phe-
 594 TAC-TCC-GAC-TGG-TAC-CAG-CCC-GCC-TGC-7GC-AAG-GCC-GGT-TGC-CAG-(ACT-TAC-AAG-TGG-GAG-
 79 tyr-ser-asp-trp-tyr-gln-pro-ala-cys-a₂-lys-ala-gly-cys-gln-thr-tyr-lys-trp-glu-

Figure 3a

654 ACC-TTC-CTG-ACC-AGC-GAG-CTG-CCG-GGG-TGG-CTG-CAG-GCC-AAC-AGG-CAC-GTC-AAG-CCC-ACC-
 99 thr-phe-leu-thr-ser-glu-leu-pro-gly-trp-leu-gln-ala-asn-arg-his-val-lys-pro-thr-
 714 GGA-AGC-GCC-GTC-GTC-GGT-CTT-TCG-ATG-GCT-GCT-TCT-TCG-GCG-CTG-ACG-CTG-GCG-ATC-TAT-
 119 gly-ser-ala-val-val-gly-leu-ser-met-ala-ala-ser-ser-ala-leu-thr-leu-ala-ile-tyr-
 774 CAC-CCC-CAG-CAG-TTC-GTC-TAC-GCG-GGA-ATG-TCG-GGC-CTG-TTG-GAC-CCC-TCC-CAG-GCG-
 139 his-pro-gln-gln-phe-val-tyr-ala-gly-ala-met-ser-gly-leu-leu-asp-pro-ser-gln-ala-
 834 ATG-GGT-CCC-ACC-CTG-ATC-GGC-CTG-GCG-ATG-GGT-GAC-GCT-GGC-TAC-AAG-GCC-TCC-GAC-
 159 met-gly-pro-thr-leu-ile-gly-leu-ala-met-gly-asp-ala-gly-gly-tyr-lys-ala-ser-asp-
 894 ATG-TGG-GGC-CCG-AAG-GAG-GAC-CCG-GCG-TGG-CAG-CGC-AAC-GAC-CCG-CTG-TTG-AAC-GTC-GGG-
 179 met-trp-gly-pro-lys-glu-asp-pro-ala-trp-gln-arg-asn-asp-pro-leu-leu-asn-val-gly-
 954 AAG-CTG-ATC-GCC-AAC-AAC-ACC-CGC-GTC-TGG-GTG-TAC-TGC-GGC-AAC-GGC-CCG-TCG-GAT-
 199 lys-leu-ile-ala-asn-asn-thr-arg-val-trp-val-tyr-cys-gly-asn-gly-lys-pro-ser-asp-
 ↓ 24
 1014 CTG-GGT-GGC-AAC-AAC-CTG-CCG-GCC-AAG-TTC-CTC-GAG-GGC-TTC-GTG-CCG-ACC-AGC-AAC-ATC-
 219 leu-gly-gly-asn-asn-leu-pro-ala-lys-phe-leu-glu-gly-phe-val-arg-thr-ser-asn-ile-
 1074 AAG-TTC-CAA-GAC-GCC-TAC-AAC-GCC-GGT-GCG-260 -CAC-AAC-GGC-GTG-TTC-GAC-TTC-CCG-GAC-
 239 lys-phe-gln-asp-ala-tyr-asn-ala-gly-gly-^a₂-his-asn-gly-val-phe-asp-phe-pro-asp-
 1134 AGC-GGT-ACG-CAC-AGC-TGG-GAG-TAC-TGG-GGC-GCG-CAG-CTC-AAC-GCT-ATG-AAG-CCC-GAC-CTG-
 259 ser-gly-thr-his-ser-trp-glu-tyr-trp-gly-ala-gln-leu-asn-ala-met-lys-pro-asp-leu-
 1194 CAA-CG -CAC-TGG-GTG-CCA-CGC-CCA-ACA-CCG-GGC-CCG-KC1-CAG-GGC-GCC-TAGCTCCGACAGACA
 279 gln-arg-^a₃ - ^b₃ - ^c₃ - ^d₃ - ^e₃ - ^f₃ -thr- ^a₄-gly-pro-^a₅ -gln-gly-ala-TER
 1258 CAACATCTAGCNCGGTGACCCCTGTGGNNCANATGTTTCTCTAAATCCCGTCCCTAGCTCCCGCNGCNCCTGTGTGTTA
 1338 GCTACCTGACNNCATGGGTTT 1358

Figure 3b

CGACACATGCCCCAGACACTGCCGGAATAATGCCACCTTCAGGCCGTGCGTGGT
 CCGGAA TTGGC CGTGAACGACCGCCGG ATAA GGGTTTCGGCGGTGCGCTTGATGCGGGT
 GGACGCCCAAGTTGTTGTTGACTACAGACACTGCCGGGCCAGCCCTGCAGTCTGACCT
 AATCAGGATGCGGCCCAACAATGCAATGATGCG TTGAGA²³ TGAGG²⁹ ATG¹⁸ AGG¹⁹ AGCA AGA
 MET-ARG-PRO → ASN-MET-HIS- GLY-CYS-VAL → GLU- MET- ARG-MET → ARG → GLU-ALA → ARG
 -59 -49 -47
 234 ATG-CAG-CTT-GTT-GAC-AGG-GTT-CGT-GGC-GCC-GTC-ACG-GGT-ATG-TCG-CGT-CGA-CTC-GTG-GTC-
 -42 MET-GLN-LEU-VAL-ASP-ARG-VAL-ARG-GLY-ALA-VAL-THR-GLY-MET-SER-ARG-ARG-LEU-VAL-VAL-
 -29
 294 GGG-GCC-GTC-GCG - CGC-CTA-GTG-TCG-GGT-CTG-GTC-GGC-GCC-GTC-GGT-GGC-ACG-GCG-ACC-GCG-
 -22 GLY-ALA-VAL-ALA - ARG-LEU-VAL-SER-GLY-LEU-VAL-GLY-ALA-VAL-GLY-GLY-THR-ALA-THR-ALA-
 354 GGG-GCA-TTT-TCC-CCG-GGC-TTG-CCG-GTG-GAG-TAC-CTG-CAG-GTG-CCG-TCG-CCG-TCG-ATG-
 -2 GLY-ALA-phe-ser-arg-pro-gly-leu-pro-val-glu-tyr-leu-gln-val-pro-ser-pro-ser-met-
 -1 +1
 414 GGC-CGT-GAC-ATC-AAG)-GTC-CAA-TTC-CAA-AGT-GGT-GGT-GCC-AAC-TCG-CCC-GCC-CTG-TAC-CTG-
 19 gly-arg-asp-ile-lys -val-gln-phe-gln-ser-gly-gly-ala-asn-ser-pro-ala-leu-tyr-leu-
 ↓ 17
 474 CTC-GAC-GGC-CTG-CCG-GCG-CAG-GAC-GAC-TTC-AGC-GGC-TGG-GAC-ATC-AAC-ACC-CCG-GCG-TTC-
 39 leu-asp-gly-leu-arg-ala-gln-asp-phe-ser-gly-trp-asp-ile-asn-thr-pro-ala-phe-
 534 GAG-TGG-TAC-GAC-CAG-TCG-GGC-CTG-TCG-GTG-GTC-ATG-CCG-GTG-GGT-GGC-CAG-TCA-AGC-TTC-
 59 glu-trp-tyr-asp-gln-ser-gly-leu-ser-val-val-met-pro-val-gly-gly-gln-ser-ser-phe-
 594 TAC-TCC-GAC-TGG-TAC-CAG-CCC-GCC-TGC-CGC-AAG-GCC-GGT-TGC-CAG-(ACT-TAC-AAG-TGG-GAG-
 79 tyr-ser-asp-trp-tyr-gln-pro-ala-cys-arg-lys-ala-gly-cys-gln- thr-tyr-lys-trp-glu-

Figure 4a

654 ACC-TTC-CTG-ACC-AGC-GAG-CTG-CCG-GGG-TGG-CTG-CAG-GCC-AAC-AGG-CAC-GTC-AAG-CCC-ACC-
 99 thr-phe-leu-thr-ser-glu-leu-pro-gly-trp-leu-gln-ala-asn-arg-his-val-lys-pro-thr-
 714 GGA-AGC-GCC-GTC-GTC-GGT-CTT-TCG-ATG-GCT-GCT-TCT-TCG-GCG-CTG-ACG-CTG-GCG-ATC-TAT-
 119 gly-ser-ala-val-gly-leu-ser-met-ala-ala-ser-ser-ala-leu-thr-leu-ala-ile-tyr-
 774 CAC-CCC-CAG-CAG-TTC-GTC-TAC-GCG-GGA-GCG-ATG-TCG-GGC-CTG-TTG-GAC-CCC-TCC-CAG-GCG-
 139 his-pro-gln-gln-phe-val-tyr-ala-gly-ala-met-ser-gly-leu-leu-asp-pro-ser-gln-ala-
 834 ATG-GGT-CCC-ACC-CTG-ATC-GGC-CTG-GCG-ATG-GGT-GAC-GCT-GGC-GGC-TAC-AAG-GCC-TCC-GAC-
 159 met-gly-pro-thr-leu-ile-gly-leu-ala-met-gly-asp-ala-gly-gly-tyr-lys-ala-ser-asp-
 894 ATG-TGG-GGC-CCG-AAG-GAG-GAC-GAC-CCG-GCG-TGG-CAG-CGC-AAC-GAC-CCG-CTG-TTG-AAC-GTC-GGG-
 179 met-trp-gly-pro-lys-glu-asp-pro-ala-trp-gln-arg-asn-asp-pro-leu-leu-asn-val-gly-
 954 AAG-CTG-ATC-GCC-AAC-AAC-ACC-CGC-GTC-TGG-GTG-TAC-TGC-GGC-AAC-GGC-CCG-TCG-GAT-
 199 lys-leu-ile-ala-asn-asn-thr-arg-val-trp-val-tyr-cys-gly-asn-gly-lys-pro-ser-asp-
 ↓ 24
 1014 CTG-GGT-GGC-AAC-AAC-CTG-CCG-GCC-AAG-TTC-CTC-GAG-GGC-TTC-GTG-CCG-ACC-AGC-AAC-ATC-
 219 leu-gly-gly-asn-asn-leu-pro-ala-lys-phe-leu-glu-gly-phe-val-arg-thr-ser-asn-ile-
 1074 AAG-TTC-CAA-GAC-GCC-TAC-AAC-GCC-GGT-GGG - CGC-CAC-AAC-GGC-GTG-TTC-GAC-TTC-CCG-GAC-
 239 lys-phe-gln-asp-ala-tyr-asn-ala-gly-gly- arg-his-asn-gly-val-phe-asp-phe-pro-asp-
 1134 AGC-GGT-ACG-CAC-AGC-TGG-GAG-TAC-TGG-GGC-GCG-CAG-CTC-AAC-GCT-ATG-AAG-CCC-GAC-CTG-
 259 ser-gly-thr-his-ser-trp-glu-tyr-trp-gly-ala-gln-leu-asn-ala-met-lys-pro-asp-leu-
 1194 CAA-CG -CAC-TGG-GTG-CCA-CGC-CCA-ACA-CCG-GGC-CCG- CCG-CAG-GGC-GCC-TAGCTCCGAACAGACA
 279 gln-arg- his-trp- val-pro-arg - pro-thr- pro-gly-pro- pro-gln-gly-ala-TER
 1258 CAACATCTAGCNCGGTGACCCCTTGTTGGNNCANATGTTTCTCTAAATCCCGTCCCTAGCTCCCGCNGCNCNCCGTGTGGTTA
 1338 GCTACCTGACNNCATGGGTTT 1358

Figure 4b

1 ACT-GCC-GGG-CCC-AGC-GCC-TGC-AGT-CTG-ACC-TAA-TTC-AGG-ATG-CGC-CCA-AAC-ATG-CAT-GGA-
 61 TGC-GTT-GAG-ATG-AGG-ATG-AGG-GAA-GCA-AGA-ATG-CAG-CTT-GTT-GAC-AGG-GTT-CGT-GGC-GCC-
 MET-GLN-LEU-VAL-ASP-ARG-VAL-ARG-GLY-ALA-
 (-43)
 121 GTC-ACG-GGT-ATG-TCG-CGT-CGA-CTC-GTG-GTC-GGC-GCC-GTC-CTA-GTG-TCG-GGT-
 -33 VAL-THR-GLY-MET-SER-ARG-ARG-LEU-VAL-VAL-GLY-ALA-VAL-GLY-ALA-LEU-VAL-SER-GLY-
 181 CTG-GTC-GGC-GCC-GTC-GGT-GGC-ACG-GCG-ACC-GCG-GGC-TTT-TCC-CGG-CGC-TTG-CCG-
 -13 LEU-VAL-GLY-ALA-VAL-GLY-GLY-THR-ALA-THR-ALA-GLY-ALA-phe-ser-arg-pro-gly-leu-pro-
 +1
 241 GTG-GAG-TAC-CTG-CAG-GTG-CCG-TCG-CCG-TCG-ATG-GGC-CGT-GAC-ATC-AAG-GTC-CAA-TTC-CAA-
 8 val-glu-tyr-leu-gln-val-pro-ser-pro-ser-met-gly-arg-asp-ile-lys-val-gln-phe-gln-
 301 AGT-GGT-GGT-GCC-AAC-TCG-CCC-GCC-CTG-TAC-CTG-CTC-GAC-GGC-CTG-CGC-GCG-CAG-GAC-GAC-
 28 ser-gly-gly-ala-asn-ser-pro-ala-leu-tyr-leu-leu-asp-gly-leu-arg-ala-gln-asp-asp-
 361 TTC-AGC-GGC-TGG-GAC-ATC-AAC-ACC-CCG-GCG-TTC-GAG-TGG-TAC-GAC-CAG-TCG-GGC-CTG-TCG-
 48 phe-ser-gly-trp-asp-ile-asn-thr-pro-ala-phe-glu-trp-tyr-asp-gln-ser-gly-leu-ser-
 421 GTG-GTC-ATG-CCG-GTG-GGT-GGC-CAG-TCA-ACC-TTC-TAC-TCC-GAC-TGG-TAC-CAG-CCC-GCC-TGC-
 68 val-val-met-pro-val-gly-gly-gln-ser-ser-phe-tyr-ser-asp-trp-tyr-gln-pro-ala-cys-
 481 GGC-AAG-GCC-GGT-TGC-CAG-ACT-TAC-AAG-TGG-GAG-ACC-TTC-CTG-ACC-AGC-GAG-CTG-CCG-GGG-
 88 gly-lys-ala-gly-cys-gln-thr-tyr-lys-trp-glu-thr-phe-leu-thr-ser-glu-leu-pro-gly-

Figure 5

541 TGG-CTG-CAG-GCC-AAC-AGG-CAC-GTC-AAG-CCC-ACC-GGA-AGC-GCC-GTC-GTC-GGT-CTT-TCG-ATG-
 108 trp-leu-gln-ala-asn-arg-his-val-lys-pro-thr-gly-ser-ala-val-gly-leu-ser-met-

 601 GCT-GCT-TCT-TCG-GCG-CTG-ACG-CTG-GCG-ATC-TAT-CAC-CCC-CAG-CAG-TTC-GTC-TAC-GCG-GGA-
 128 ala-ala-ser-ser-ala-leu-thr-leu-ala-ile-tyr-his-pro-gln-gln-phe-val-tyr-ala-gly-

 661 GCG-ATG-TCG-GGC-CTG-TTG-GAC-CCC-TCC-CAG-GCG-ATG-GGT-CCC-ACC-CTG-ATC-GGC-CTG-GCG-
 148 ala-met-ser-gly-leu-leu-asp-pro-ser-gln-ala-met-gly-pro-thr-leu-ile-gly-leu-ala-

 721 ATG-GGT-GAC-GCT-GGC-GGC-TAC-AAG-GCC-TCC-GAC-ATG-TGG-GGC-CCG-AAG-GAG-GAC-CCG-GCG-
 168 met-gly-asp-ala-gly-gly-tyr-lys-ala-ser-asp-met-trp-gly-pro-lys-glu-asp-pro-ala-

 781 TGG-CAG-CGC-AAC-GAC-CCG-CTG-TTG-AAC-GTC-GGG-AAG-CTG-ATC-GCC-AAC-AAC-ACC-CGC-GTC-
 188 trp-gln-arg-asn-asp-pro-leu-leu-leu-asn-val-gly-lys-leu-ile-ala-asn-asn-thr-arg-val-

 841 TGG-GTG-TAC-TGC-GGC-AAC-GGC-AAG-CCG-TCG-GAT-CTG-GGT-GGC-AAC-AAC-CTG-CCG-GCC-AAG-
 208 trp-val-tyr-cys-gly-asn-gly-lys-pro-ser-asp-leu-gly-gly-asn-asn-leu-pro-ala-lys-

 901 TTC-CTC-GAG-GGC-TTC-GTG-CGG-ACC-AGC-AAC-ATC-AAG-TTC-CAA-GAC-GCC-TAC-AAC-GCC-GGT-
 228 phe-leu-glu-gly-phe-val-arg-thr-ser-asn-ile-lys-phe-gln-asp-ala-tyr-asn-ala-gly-

 961 GGC-GGC-CAC-AAC-GGC-GTG-TTC-GAC-TTC-CCG-GAC-AGC-GGT-ACG-CAC-AGC-TGG-GAG-TAC-TGG-
 248 gly-gly-his-asn-gly-val-phe-asp-phe-pro-asp-ser-gly-thr-his-ser-trp-glu-tyr-trp-

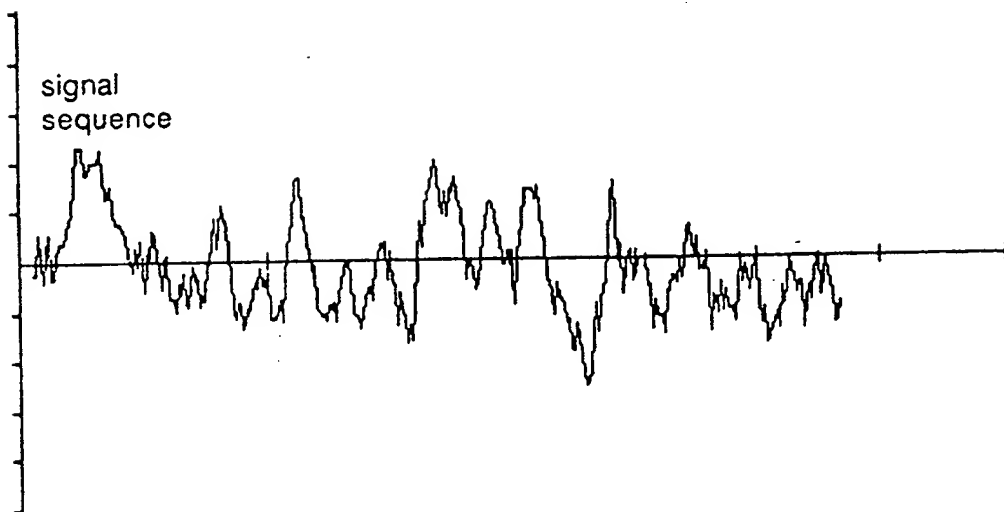
Figure 5 (con't)

1021 GG[C]-GCG-CAG-CTC-AAC-GCT-ATG-AAG-CCC-GAC-CTG-CAA-CGG-GCA-CTG-GGT-GCC-ACG-CCC-AAC-
 268 gly-ala-gln-leu-asn-ala-met-lys-pro-asp-leu-gln-arg-ala-leu-gly-ala-thr-pro-asn-
 1081 ACC-GGG-CCC-GCG-CCC-CAG-GGC-GCC-TAG-CTC-CGA-ACA-GAC-ACA-ACA-TCT-AGC-GGC-GGT-GAC-
 288 thr-gly-pro-ala-pro-gln-gly-ala-TER
 (1104)
 (295)
 1141 CCT-TGT-GGT-CGC-CGT-AGA-TGT-TTC-CTA-AAT-CCC-GTC-CCT-AGC-TCC-CGC-CGC-GGG-CCG-
 1201 TGT-GGT-TAG-CTA-CCT-GAC-GGG-CTA-GGG-GTT-GGC-CGG-GGT-TGA-CGC-CGG-GTG-CAC-ACA-
 1261 GCC-TAC-ACG-AAC-GGA-AGG-TGG-ACA-CAT-GAA-GGG-TCG-GTC
 (1299)

Figure 5 (con't)

Hydropathy

M. tuberculosis 32 kD protein



BCG α -antigen

Hydropathy

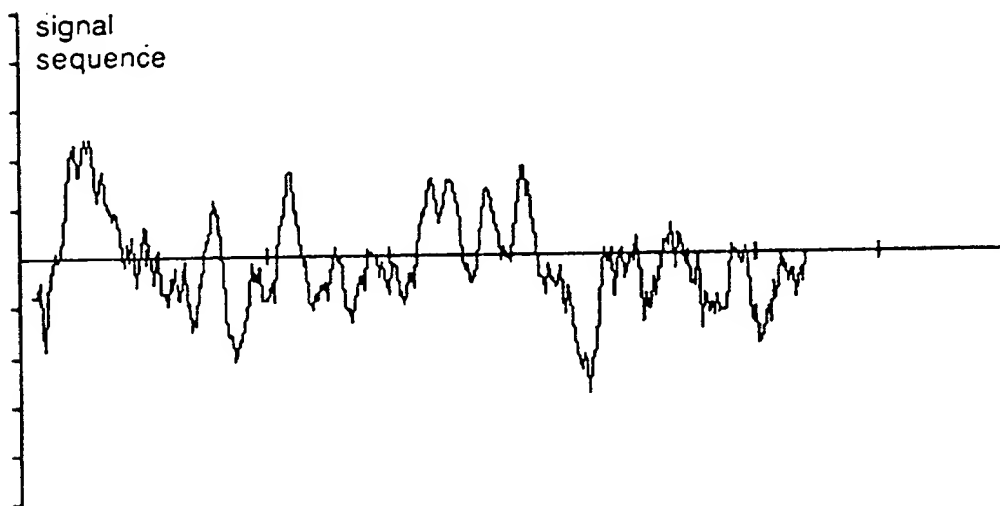


Fig. 6

Fig. 7

neu eingereicht / Newly filed
Nouvellement déposé

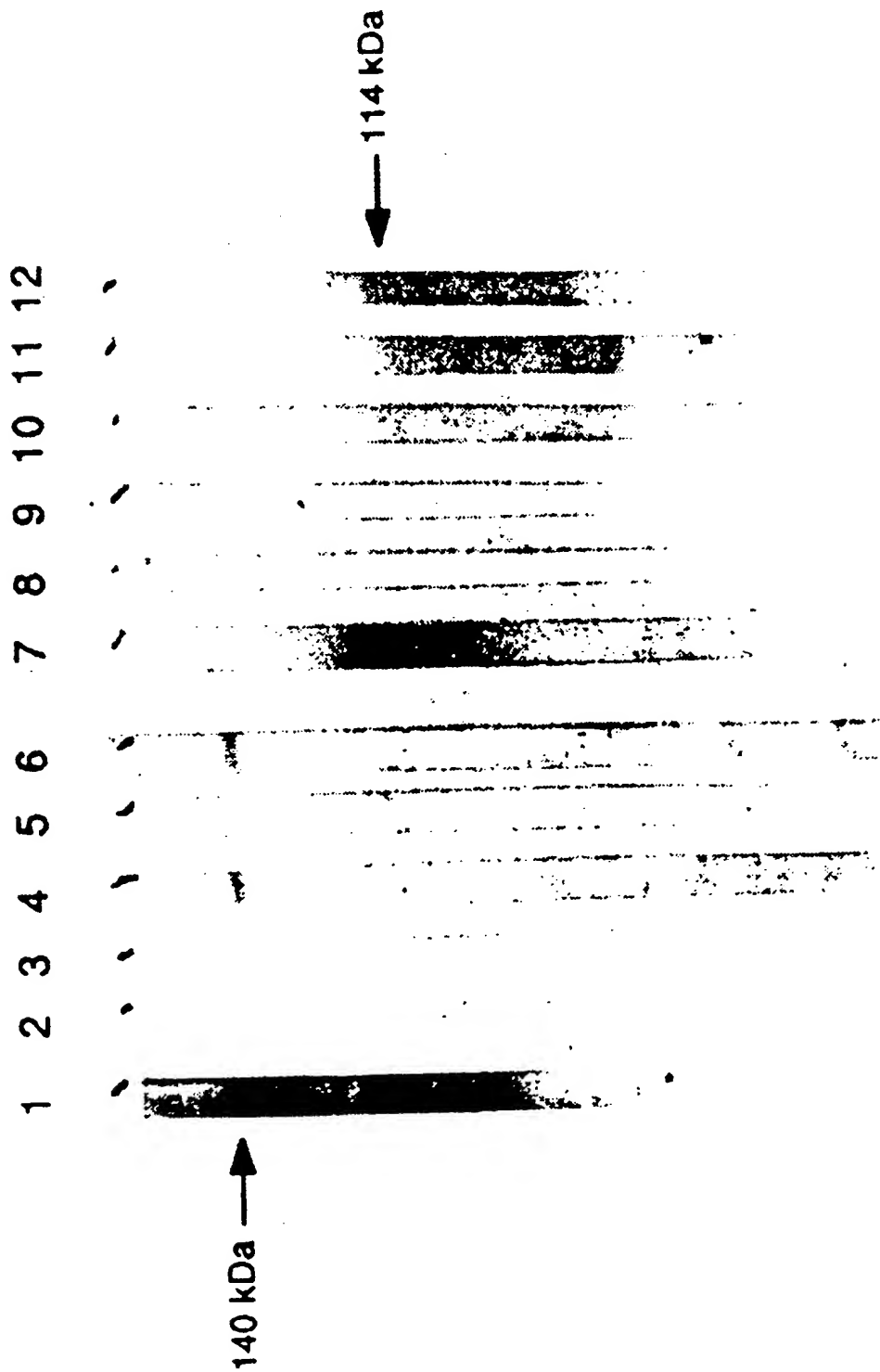


Figure 8

1 ATG CAGCTTGTTGACAGGGTTCGTGGCGCCGTCACGGGTATGTCGCCTCGACTC
||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
1 ATG CAGCTTGTTGACAGGGTTCGTGGCGCCGTCACGGGTATGTCGCCTCGACTC
||| || | | | | | | | | | |
1 ATG ACAGACGTGAGCCGAAAGATTCTGAG CTT GGGGACGCCG ATTGA TG

[illegible]

		P1
110	GCA CGGCGACCGCGGGGGGCATTTTCCCGGCCGGGCTTGCCGGTG	GAGTACCTG
107	GCA CGGCGACCGCGGGGGGCATTTTCCCGGCCGGGCTTGCCGGTG	GAGTACCTG
101	GAG CGGCAACCGCGGGGCGCGTTCTCCCGGCCGGGGCTGCCGGTC	GAGTACCTG

163	CAGGTGCCGTCGCCGTCGATGGGCCG	TGACATCAAGGTCCAATTCCAAAGTGGT
160	CAGGTGCCGTCGCCGTCGATGGGCCG	TGACATCAAGGTCCAATTCCAAAGTGGT
154	CAGGTGCCGTCGCCGTCGATGGGCCG	CGACATCAAGGTTCAAGTCCAGAGCGGT

217 GGTGCCAAC TCGCCCGCCCTGTACCTG CTCGACGGCCTGCGCGCGCAGGACGA
||||| |||||||
214 GGTGCCAAC TCGCCCGCCCTGTACCTG CTCGACGGCCTGCGCGCGCAGGACGA
|| ||| || ||| ||| ||||||| ||| |||||
208 GGAACAAC TCACCTGCGGTTTATCTG CTCGACGGCCTGCGCGCCCAAGACGA

Figure 9b

270 CTCAGCGGCTGGGAC ATCAACACCCCGGCGTTCGAGTGGTAC GACCAGTCGG
 |||||
 267 CTCAGCGGCTGGGAC ATCAACACCCCGGCGTTCGAGTGGTAC GACCAGTCGG
 || || |||||
 261 CTACAACGGCTGGGAT ATCAACACCCCGGCGTTCGAGTGGTAC TACCAGTCGG
 |||||

323 GCCTGTCGGTGGTCATGCCGGTGGGTGGCCAGTCAAGCTTCTACTCCGACTGGTA
 |||||
 320 GCCTGTCGGTGGTCATGCCGGTGGGTGGCCAGTCAAGCTTCTACTCCGACTGGTA
 | ||||| | ||||| || || ||||| |||||
 314 GACTGTCGATAGTCATGCCGGTCGGCGGGCAGTCCAGCTTCTACAGCGACTGGTA

378 CCAGCCCGCCTGCCGCAAGGCCGGT TGCCAGACTTACAAGTGGGA GACCT TC
 |||||
 375 CCAGCCCGCCTGCCGCAAGGCCGGT TGCCAGACTTACAAGTGGGA GACCT TC
 | || ||||| | ||||| || |||||
 369 CAGCCCGCCTGCCGTAAGGCTGGC TGCCAGACTTACAAGTGGGA AACCC TC

430 CTGACCAGCGAGCTGCCG GGGTGGCTGCAGGCCAACAGGCACGTCAAGCCCACC
 |||||
 427 CTGACCAGCGAGCTGCCG GGGTGGCTGCAGGCCAACAGGCACGTCAAGCCCACC
 ||||| || || ||||| || |||||
 421 CTGACCAGCGAGCTGCCG CAATGGTTGTCCGCCAACAGGGCCGTGAAGCCCACC

484 GGAAGCGCCGTCGTCGGTCTTTCGATGGCTGCTTCTTCG GCGCTGACGCTGGCG
 |||||
 481 GGAAGCGCCGTCGTCGGTCTTTCGATGGCTGCTTCTTCG GCGCTGACGCTGGCG
 || ||||| | |||| | ||||| || || || |||||
 475 GGCAGCGCTGCAATCGGCTTGTGATGGCCGGCTCGTCG GCAATGATCTTGGCC

PROBE REGION C

Figure 9c

538 ATCTATC ACCCCCAGCAGTTCGTCTACGCCGGAGCGATGTCGGGCCTGTTGGAC
 ||||| ||||||||||||||||||||||||||||||||||||||||
 535 ATCTATC ACCCCCAGCAGTTCGTCTACGCCGGAGCGATGTCGGGCCTGTTGGAC
 ||| ||||||||||||||| ||||| || ||||| ||||| |||||
 529 GCCTACC ACCCCCAGCAGTTCATCTACGCCGGCTCGCTGTCGGCCCTGCTGGAC

P5

592 CCCTCCCAGGCGATGGGTCCAC CCTGATCGGCCTGGCGATGGGTGACGC TGG
 ||||| ||||||||||||||||||| ||||||||||||||||||| |||
 589 CCCTCCCAGGCGATGGGTCCAC CCTGATCGGCCTGGCGATGGGTGACGC TGG
 ||||| ||||| ||||| ||||||||||||||| ||||| ||||| |||
 583 CCCTCTCAGGGGATGGG CCTGATCGGCCTCGCGATGGGTGACGC CGG

645 CGGCTACAAGGCCTCCGACATGTGGGGCCCGAAGGAGGACCCGGCGTGGCAGCGC
 ||||||||||||||||||||||||||||||||||||||||||||
 642 CGGCTACAAGGCCTCCGACATGTGGGGCCCGAAGGAGGACCCGGCGTGGCAGCGC
 ||| ||||||||| | ||||||||| || | ||||||| ||| |||||
 631 CGGTTACAAGGCCGAGACATGTGGGGTCCCTCGAGTGACCCGGCATGGGAGCGC

PROBE REGION D

700 AACGAC CCGCTGTTGAACGTCGGGAAG CTGATCGCCAACAACACCCGCGTCTG
 ||||| ||||||||||||||||||| |||||||||||||||||||
 697 AACGAC CCGCTGTTGAACGTCGGGAAG CTGATCGCCAACAACACCCGCGTCTG
 ||||| || | | | || ||||| ||||| ||||| ||||| |||
 686 AACGAC CCTACGCAGCAGATCCCCAAG CTGGTCGCAAACAACACCCGGCTATG

PROBE REGION E

753 GGTGTACTGCGGCAACGGC AAGCCGTCGGATCTGGGTGGCAAC AACCTGCCGG
 ||||||||||||||||| ||||||||||||||||||| |||||||||
 750 GGTGTACTGCGGCAACGGC AAGCCGTCGGATCTGGGTGGCAAC AACCTGCCGG
 ||| || ||||| ||||| | ||| || ||||| || | ||| |||
 739 GGTTTATTGCGGGAACGGC ACCCCGAACGAGTTGGGCGGTGCC AACATACCCG

		P6
861	CAACGCCGGTGGCGGCCACAACGGCGTGTTGACTTCCGGACAGCGGT 	ACGCA
858	CAACGCCGGTGGCGGCCACAACGGCGTGTTGACTTCCGGACAGCGGT 	ACGCA
847	CAAGCCCGCGGGCGGGCACAACGCCGTGTTCAACTTCCGCCCAACGGC	ACGCA

915 CAGCTGGGAGTACTGGGGCGC GCAGCTCAACGCTATGAAGCCCGACCTGCA AC
|||||
912 CAGCTGGGAGTACTGGGGCGC GCAGCTCAACGCTATGAAGCCCGACCTGCA AC
|||||
901 CAGCTGGGAGTACTGGGGCGC TCAGCTCAACGCCATGAAGGGTGACCTGCAGAG

```

PROBE REGION F
968  GGGCACTGGGTGCCACGCCCAACACCGGGCCCCGCGCCCCAGGG CGCCTAG
      ||||||||||||||||||||||||||||| |||| ||||| |||||||
965  GGGCACTGGGTGCCACGCCCAACACCGGGCC CGCCGCAGGG CGCCTAG
      | | || || | |
955  TTCGTTAGGCGCC GGCTGA

```

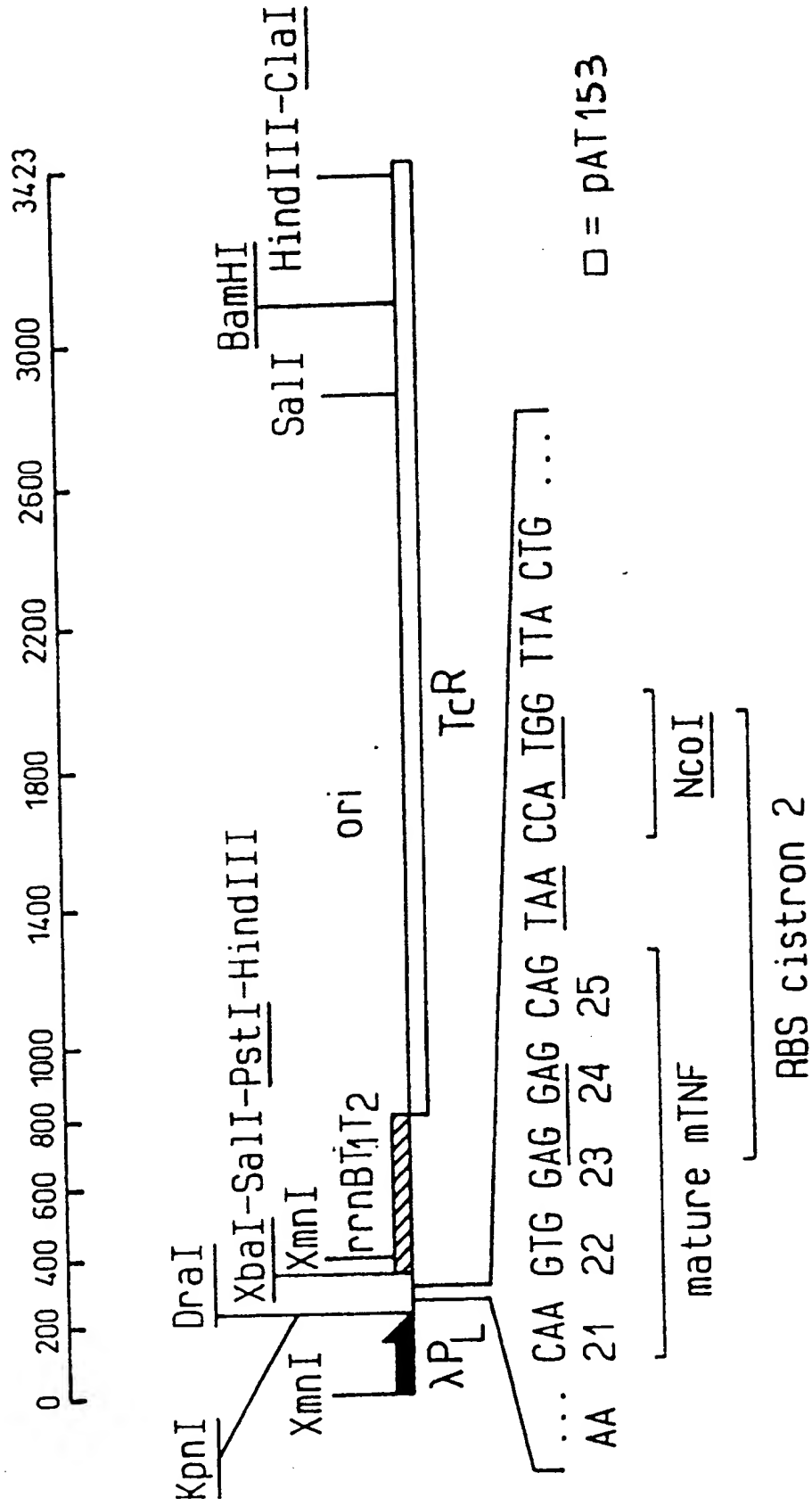


FIG. 10a

From: PIGRI

Fig. 10b

3	9	15	21	27	33	39	45
1	TTC CGG GGA TCT CTC ACC TAC CAA ACA ATG CCC	TGC	AAA	AAA	AAA	AAA	AAA
AAG GCC CCT AGA GAG TGG ATG GTT TGT TAC GGG	GGG	ACG	TTT	TTT	TTT	TTT	TTT
46	TAA ATT CAT ATA AAA AAC ATA CAG ATA ACC ATC TGC	GGT	GAT	AAA	AAA	AAA	AAA
ATT TAA GTA TAT TTT TTG TAT GTC TAT TGG TAG ACG	CCA	CTA	TTT	TTT	TTT	TTT	TTT
91	TTA TCT CTG GCG GTG TCG ACA TAA ATA CCA CTG GCG	GAT	ATA	CTG	ATA	CTG	CTG
AAT AGA GAC CGC CAC AAC TGT ATT TAT TAT GGT GAC	CGC	TAT	GAT	GAC	TAT	GAC	GAC
136	AGC ACA TCA GCA GGA GGC ACT GAC CAC CAT GAA GGT	GAC	GCT	CTT	GCT	CTT	CTT
TCG TGT AGT CGT CCT GCG TGA CTG GTG TTA CTT CCA	CTG	GAA	GAA	GAA	GAA	GAA	GAA
181	AAA AAT TAA GCC CTG AAG AAC GGC AGG GGT ACC	AGG	TTT	AAA	TTT	AAA	AAA
TTT TTA ATT CGG GAC TTC TTC CCG TCC TCC TCC	TCC	AAA	TTT	TTT	TTT	TTT	TTT
226	TCA TGG TAA GAT CAA GTA GTC AAA ATT CGA GTG	ACA	CTG	TAG	CTG	TAG	TAG
AGT ACC ATT CTA GTT CAT CAG TTT TAA GCT CAC	TGT	GAC	GAC	ATC	GAC	ATC	ATC
271	CCC ACG TCG TAG CAA ACC ACC AAC TGG TGG	AGT	CAT	GGT	CAT	GGT	GGT
GGG TGC AGC ATC GTT GGT TGC TGC TCA TTG	GTA	CCA	CCA	CCA	CCA	CCA	CCA
316	TAC TGG AGA AGG GGG ACC AAC TCA GCG CTG	AGG	TGC	CCA	TGC	CCA	CCA
ATG ACC TCT TCC CCC TGG TTG AGT CGC GAC	TAG	ACG	GGT	GGT	GGT	GGT	GGT

Fig. 10b (Con't)

361	AGT	CTA	GAG	TCG	ACC	TGC	AGC	CCA	AGC	TTG	GCT	GTT	TTG	GCG	GAT
	TCA	GAT	CTC	AGC	TGG	ACG	TCG	GGT	TCG	AAC	CGA	CAA	AAC	CGC	CTA
406	GAG	AGA	AGA	TTT	TCA	GCC	TGA	TAC	AGA	TTA	AAT	CAG	AAC	GCA	GAA
	CTC	TCT	TCT	AAA	AGT	CGG	ACT	ATG	TCT	AAT	TTA	GTC	TTG	CGT	CTT
451	GCG	GTC	TGA	TAA	AAC	AGA	ATT	TGC	CTG	GCG	GCA	GTA	GCG	CGG	TGG
	CGC	CAG	ACT	ATT	TTG	TCT	TAA	ACG	GAC	CGC	CGT	CAT	CGC	GCC	ACC
496	TCC	CAC	CTG	ACC	CCA	TGC	CGA	ACT	CAG	AAG	TGA	AAC	GCC	GTA	GCG
	AGG	GTG	GAC	TGG	GGT	ACG	GCT	TGA	GTC	TTC	ACT	TTG	CGG	CAT	CGC
541	CCG	ATG	GTA	GTG	TGG	GGT	CTC	CCC	ATG	CGA	GAG	TAG	GGA	ACT	GCC
	GGC	TAC	CAT	CAC	ACC	CCA	GAG	GGG	TAC	GCT	CTC	ATC	CCT	TGA	CGG
586	AGG	CAT	CAA	ATA	AAA	CGA	AAG	GCT	CAG	TCG	AAA	GAC	TGG	GCC	TTT
	TCC	GTA	GTT	TAT	TTT	GCT	TTC	CGA	GTC	AGC	TTT	CTG	ACC	CGG	AAA
631	CGT	TTT	ATC	TGT	TGT	TTG	TCG	GTG	AAC	GCT	CTC	CTG	AGT	AGG	ACA
	GCA	AAA	TAG	ACA	ACA	AAC	AGC	CAC	TTG	CGA	GAG	GAC	TCA	TCC	TGT
676	AAT	CCG	CCG	GGA	GCG	GAT	TTG	AAC	GTT	GCG	AAG	CAA	CGG	CCC	GGA
	TTA	GGC	GGC	CCT	CGC	CTA	AAC	TTG	CAA	CGC	TTT	GTT	GCC	GGG	CCT
721	GGG	TGG	CGG	GCA	GGA	CGC	CCG	CCA	TAA	ACT	GCC	AGG	CAT	CAA	ATT
	CCC	ACC	GCC	CGT	CCT	GCG	GGT	ATT	TGA	TGA	CGG	TCC	GTA	GTT	TAA

Fig. 10b (Con't)

766	AAG	CAG	AAG	GCC	ATC	CTG	ACG	GAT	GGC	CTT	TTT	GCG	TTT	CTA	CAA
	TTC	GTC	TTC	CGG	TAG	GAC	TGC	CTA	CCG	GAA	AAA	CGC	AAA	GAT	GTT
811	ACT	CTT	TTG	TTT	ATT	TTT	CTA	AAT	ACA	TTC	AAA	TAT	GTA	TCC	GCT
	TGA	GAA	AAC	AAA	TAA	AAA	GAT	TTA	TGT	AAG	TTT	ATA	CAT	AGG	CGA
856	CAT	GAG	ACA	ATA	ACC	CTG	ATA	AAT	GCT	TCA	ATA	ATA	AAA	GGA	TCT
	GTA	CTC	TGT	TAT	TGG	GAC	TAT	TTA	CGA	AGT	TAT	TAT	TTT	CCT	AGA
901	AGG	TGA	AGA	TCC	TTT	TTG	ATA	ATC	TCA	TGA	CCA	AAA	TCC	CTT	AAC
	TCC	ACT	TCT	AGG	AAA	AAC	TAT	TAG	AGT	ACT	GGT	TTT	AGG	GAA	TTG
946	GTG	AGT	TTT	CGT	TCC	ACT	GAG	CGT	CAG	ACC	CCG	TAG	AAA	AGA	TCA
	CAC	TCA	AAA	GCA	AGG	TGA	CTC	GCA	GTC	TGG	GGC	ATC	TTT	TCT	AGT
991	AAG	GAT	CTT	CTT	GAG	ATC	CTT	TTT	TTC	TGC	GCG	TAA	TCT	GCT	GCT
	TTC	CTA	GAA	GAA	CTC	TAG	GAA	AAA	AAG	ACG	CGC	ATT	AGA	CGA	CGA
1036	TGC	AAA	CAA	AAA	AAC	CAC	CGC	TAC	CAG	CGG	TGG	TTT	GTT	TGC	CGG
	ACG	TTT	GTT	TTT	TTG	GTG	GCG	ATG	GTC	GCC	ACC	AAA	CAA	ACG	GCC

Fig. 10b (Con't)

1081	ATC	AAG	AGC	TAC	CAA	CTC	TTT	TTC	CGA	AGG	TAA	CTG	GCT	TCA	GCA
	TAG	TTC	TCG	ATG	GTT	GAG	AAA	AAG	GCT	TCC	ATT	GAC	CGA	AGT	CGT
1126	GAG	CGC	AGA	TAC	CAA	ATA	CTG	TCC	TTC	TAG	TGT	AGC	CGT	AGT	TAG
	CTC	GCG	TCT	ATG	GTT	TAT	GAC	AGG	AAG	ATC	ACA	TCG	GCA	TCA	ATC
1171	GCC	ACC	ACT	TCA	AGA	ACT	CTG	TAG	CAC	CGC	CTA	CAT	ACC	TCG	CTC
	CGG	TGG	TGA	AGT	TCT	TGA	GAC	ATC	GTG	GCG	GAT	GTA	TGG	AGC	GAG
1216	TGC	TAA	TCC	TGT	TAC	CAG	TGG	CTG	CTG	CCA	GTG	GCG	ATA	AGT	CGT
	ACG	ATT	AGG	ACA	ATG	GTC	ACC	GAC	GAC	GGT	CAC	CGC	TAT	TCA	GCA
1261	GTC	TTA	CCG	GGT	TGG	ACT	CAA	GAC	GAT	AGT	TAC	CGG	ATA	AGG	CGC
	CAG	AAT	GGC	CCA	ACC	TGA	GTT	CTG	CTA	TCA	ATG	GCC	TAT	TCC	GCG
1306	AGC	GGT	CGG	GCT	GAA	CGG	GGG	GTT	CGT	GCA	CAC	AGC	CCA	GCT	TGG
	TCG	CCA	GCC	CGA	CTT	GCC	CCC	CAA	GCA	CGT	GTG	TCG	GGT	CGA	ACC
1351	AGC	GAA	CGA	CCT	ACA	CCG	AAC	TGA	GAT	ACC	TAC	AGC	GTG	AGC	ATT
	TCG	CTT	GCT	GGA	TGT	GGC	TTG	ACT	CTA	TGG	ATG	TCG	CAC	TCG	TAA

Fig. 10b (Con't)

1396	GAG	AAA	GCG	CCA	CGC	TTC	CCG	AAG	GGA	GAA	AGG	CGG	ACA	GGT	ATC
	CTC	TTT	CGC	GGT	GCG	AAG	GGC	TTC	CCT	CTT	TCC	GCC	TGT	CCA	TAG
1441	CGG	TAA	GCG	GCA	GGG	TCG	GAA	CAG	GAG	AGC	GCA	CGA	GGG	AGC	TTC
	GCC	ATT	CGC	CGT	CCC	AGC	CTT	GTC	CTC	TCG	CGT	GCT	CCC	TCG	AAG
1486	CAG	GGG	GAA	ACG	CCT	GGT	ATC	TTT	ATA	GTC	CTG	TCG	GGT	TTC	GCC
	GTC	CCC	CTT	TGC	GGA	CCA	TAG	AAA	TAT	CAG	GAC	AGC	CCA	AAG	CGG
1531	ACC	TCT	GAC	TTG	AGC	GTC	GAT	TTT	TGT	GAT	GCT	CGT	CAG	GGG	GGC
	TGG	AGA	CTG	AAC	TCG	CAG	CTA	AAA	ACA	CTA	CGA	GCA	GTC	CCC	CCG
1576	GGA	GCC	TAT	GGA	AAA	ACG	CCA	GCA	ACG	CGG	CCT	TTT	TAC	GGT	TCC
	CCT	CGG	ATA	CCT	TTT	TGC	GGT	CGT	TGC	GCC	GGA	AAA	ATG	CCA	AGG

Fig. 10b (Con't)

1621	TGG	CCT	TTT	GCT	GGC	CTT	TTG	CTC	ACA	TGT	ACA	TGT	TCT	TTC	CTG	CGT	TAT
	ACC	GGA	AAA	CGA	CCG	GAA	AAC	GAG	TGT	ACA	AGA	AAG	GAC	GCA	ATA		
1666	CCC	CTG	ATT	CTG	TGG	ATA	ACC	GTA	TTA	CCG	CCT	TTG	AGT	GAG	CTG		
	GGG	GAC	TAA	GAC	ACC	TAT	TGG	CAT	AAT	GGC	GGA	AAC	TCA	CTC	GAC		
1711	ATA	CCG	CTC	GCC	GCA	GCC	GAA	CGA	CCG	AGC	GCA	GGC	AGT	CAG	TGA		
	TAT	GGC	GAG	CGG	CGT	CGG	CTT	GCT	GGC	TCG	CGT	CGC	TCA	GTC	ACT		
1756	GCG	AGG	AAG	CGG	AAG	AGC	GCT	GAC	TTC	CGC	GTT	TCC	AGA	CTT	TAC		
	CGC	TCC	TTC	GCC	TTC	TCG	CGA	CTG	AAG	GCG	CAA	AGG	TCT	GAA	ATG		
1801	GAA	ACA	CGG	AAA	CCG	AAG	ACC	ATT	CAT	GTT	GTT	GCT	CAG	GTC	GCA		
	CTT	TGT	GCC	TTT	GGC	TTC	TGG	TAA	GTA	CAA	CAA	CGA	GTC	CAG	CGT		
1846	GAC	GTT	TTG	CAG	CAG	CAG	TCG	CTT	CAC	GTT	CGC	TCG	CGT	ATC	GGT		
	CTG	CAA	AAC	GTC	GTC	GTC	AGC	GAA	GTG	CAA	GCG	AGC	GCA	TAG	CCA		
1891	GAT	TCA	TTC	TGC	TAA	CCA	GTA	AGG	CAA	CCC	CGC	CAG	CCT	AGC	CGG		
	CTA	AGT	AAG	ACG	ATT	GGT	CAT	TCC	GTT	GGG	GCG	GTC	GGA	TCG	GCC		

Fig. 10b (Con't)

1936	GTC	CTC	AAC	GAC	AGG	AGC	ACG	ATC	ATG	CGC	ACC	CGT	GGC	CAG	GAC
	CAG	GAG	TTG	CTG	TCC	TCG	TGC	TAG	TAC	GCG	TGG	GCA	CCG	GTC	CTG
1981	CCA	ACG	CTG	CCC	GAG	ATG	CGC	CGC	GTG	CGG	CTG	CTG	GAG	ATG	GCG
	GGT	TGC	GAC	GGG	CTC	TAC	GCG	GCG	CAC	GCC	GAC	GAC	CTC	TAC	CGC
2026	GAC	GCG	ATG	GAT	ATG	TTC	TGC	CAA	GGG	TTG	GTT	TGC	GCA	TTC	ACA
	CTG	CGC	TAC	CTA	TAC	AAG	ACG	GTT	CCC	AAC	CAA	ACG	CGT	AAG	TGT
2071	GTT	CTC	CGC	AAG	AAT	TGA	TTG	GCT	CCA	ATT	CTT	GGA	GTG	GTG	AAT
	CAA	GAG	GCG	TTC	TTA	ACT	AAC	CGA	GGT	TAA	GAA	CCT	CAC	CAC	TTA
2116	CCG	TTA	GCG	AGG	TGC	CGC	CGG	CTT	CCA	TTC	AGG	TCG	AGG	TGG	CCC
	GGC	AAT	CGC	TCC	ACG	GCG	GCC	GAA	GGT	AAG	TCC	AGC	TCC	ACC	GGG
2161	GGC	TCC	ATG	CAC	CGC	GAC	GCA	ACG	CGG	GGA	GGC	AGA	CAA	GGT	ATA
	CCG	AGG	TAC	GTG	GCG	CTG	CGT	TGC	GCC	CCT	CCG	TCT	GTT	CCA	TAT
2206	GGG	CGG	CGC	CTA	CAA	TCC	ATG	CCA	ACC	CGT	TCC	ATG	TGC	TCG	CCG
	CCC	GCC	GCG	GAT	GTT	AGG	TAC	GGT	TGG	GCA	AGG	TAC	ACG	AGC	GGC

Fig. 10b (Con't)

2251	AGG	CGG	CAT	AAA	TCG	CCG	TGA	CGA	TCA	GGC	GTC	CAG	TGA	TCG	AAG
	TCC	GCC	GTA	TTT	AGC	GGC	ACT	GCT	AGT	CGC	CAG	GTC	ACT	AGC	TTC
2296	TTA	GGC	TGG	TAA	GAG	CCG	CGA	GGC	ATC	CTT	GAA	GCT	GTC	CCT	GAT
	AAT	CCG	ACC	ATT	CTC	GGC	GCT	CGC	TAG	GAA	CTT	CGA	CAG	GGA	CTA
2341	GGT	CGT	CAT	CTA	CCT	GCC	TGG	ACA	GCA	TGG	CCT	GCA	ACG	CGG	GCA
	CCA	GCA	GTA	GAT	GGA	CGG	ACC	TGT	CGT	ACC	GGA	CGT	TGC	GCC	CGT
2386	TCC	CGA	TGC	CGC	CGG	AAG	CGA	GAA	GAA	TCA	TAA	TGG	GGA	AGG	CCA
	AGG	GCT	ACG	GCG	GCC	TTC	GCT	CTT	CTT	AGT	ATT	ACC	CCT	TCC	GGT
2431	TCC	AGC	CTC	GCG	TCG	CGA	ACG	CCA	GCA	AGA	CGT	AGC	CCA	GCG	CGT
	AGG	TCG	GAG	CGC	AGC	GCT	TGC	GGT	CGT	TCT	GCA	TCG	GGT	CGC	GCA

Fig. 10b (Con't)

2476	CGG	CCG	CCA	TGC	CGG	CGA	TAA	TGG	CCT	GCT	TCT	CGC	CGA	AAC	GTT
	GCC	GGC	GGT	ACG	GCC	GCT	ATT	ACC	GGA	CGA	AGA	GCG	GCT	TTG	CAA
2521	TGG	TGG	CGG	GAC	CAG	TGA	CGA	AGG	CTT	GAG	CGA	GGG	CGT	GCA	AGA
	ACC	ACC	GCC	CTG	GTC	ACT	GCT	TCC	GAA	CTC	GCT	CCC	GCA	CGT	TCT
2566	TTC	CGA	ATA	CCG	CAA	GCG	ACA	GGC	CGA	TCA	TCG	TCG	CGC	TCC	AGC
	AAG	GCT	TAT	GGC	GTT	CGC	TGT	CCG	GCT	AGT	AGC	AGC	GCG	AGG	TCG
2611	GAA	AGC	GGT	CCT	CGC	CGA	AAA	TGA	CCC	AGA	GCG	CTG	CCG	GCA	CCT
	CTT	TCG	CCA	GGA	GCG	GCT	TTT	ACT	GGG	TCT	CGC	GAC	GGC	CGT	GGA
2656	GTC	CTA	CGA	GTT	GCA	TGA	TAA	AGA	AGA	CAG	TCA	TAA	GTG	CGG	CGA
	CAG	GAT	GCT	CAA	CGT	ACT	ATT	TCT	TCT	GTC	AGT	ATT	CAC	GCC	GCT
2701	CGA	TAG	TCA	TGC	CCC	GCG	CCC	ACC	GGA	AGG	AGC	TGA	CTG	GGT	TGA
	GCT	ATC	AGT	ACG	GGG	CGC	GGG	TGG	CCT	TCC	TCG	ACT	GAC	CCA	ACT
2746	AGG	CTC	TCA	AGG	GCA	TCG	GTC	GAC	GCT	CTC	CCT	TAT	GCG	ACT	CCT
	TCC	GAG	AGT	TCC	CGT	AGC	CAG	CTG	CGA	GAG	GGA	ATA	CGC	TGA	GGA

Fig. 10b (Con't.)

2791	GCA	TTA	GGA	AGC	AGC	CCA	GTA	GTA	GGT	TGA	GGC	CGT	TGA	GCA	CCG
	CGT	AAT	CCT	TCG	TCG	GGT	CAT	CAT	CCA	ACT	CCG	GCA	ACT	CGT	GGC
2836	CCG	CCG	CAA	GGA	ATG	GTG	CAT	GCA	AGG	AGA	TGG	CGC	CCA	ACA	GTC
	GGC	GGC	GTT	CCT	TAC	CAC	GTA	CGT	TCC	TCT	ACC	GCG	GGT	TGT	CAG
2881	CCC	CGG	CCA	CGG	GGC	CTG	CCA	CCA	TAC	CCA	CGC	CGA	AAC	AAG	CGC
	GGG	GCC	GGT	GCC	CCG	GAC	GGT	GGT	ATG	GGT	GCG	GCT	TTG	TTC	GCG
2926	TCA	TGA	GCC	CGA	AGT	GGC	GAG	CCC	GAT	CTT	CCC	CAT	CGG	TGA	TGT
	AGT	ACT	CGG	GCT	TCA	CCG	CTC	GGG	CTA	GAA	GGG	GTA	GCC	ACT	ACA
2971	CGG	CGA	TAT	AGG	CGC	CAG	CAA	CCG	CAC	CTG	TGG	CGC	CGG	TGA	TGC
	GCC	GCT	ATA	TCC	GCG	GTC	GTT	GGC	GTG	GAC	ACC	GCG	GCC	ACT	ACG
3016	CGG	CCA	CGA	TGC	GTC	CGG	CGT	AGA	GGA	TCC	ACA	GGA	CGG	GTG	TGG
	GCC	GGT	GCT	ACG	CAG	GCC	GCA	TCT	CCT	AGG	TGT	CCT	GCC	CAC	ACC
3061	TCG	CCA	TGA	TCG	CGT	AGT	CGA	TAG	TGG	CTC	CAA	GTA	GCG	AAG	CGA
	AGC	GGT	ACT	AGC	GCA	TCA	GCT	ATC	ACC	GAG	GTT	CAT	CGC	TTC	GCT

Fig. 10b (Con't)

3106	GCA	GGA	CTG	GGC	GGC	GGC	CAA	AGC	GGT	CGG	ACA	GTG	CTC	CGA	GAA
	CGT	CCT	GAC	CCG	CCG	CCG	GTT	TCG	CCA	GCC	TGT	CAC	GAG	GCT	CTT
3151	CGG	GTG	CGC	ATA	GAA	ATT	GCA	TCA	ACG	CAT	ATA	GCG	CTA	GCA	GCA
	GCC	CAC	GCG	TAT	CTT	TAA	CGT	AGT	TGC	GTA	TAT	CGC	GAT	CGT	CGT
3196	CGC	CAT	AGT	GAC	TGG	CGA	TGC	TGT	CGG	AAT	GGA	CGA	TAT	CCC	GCA
	GCG	GTA	TCA	CTG	ACC	GCT	ACG	ACA	GCC	TTA	CCT	GCT	ATA	GGG	CGT
3241	AGA	GGC	CCG	GCA	GTA	CCG	GCA	TAA	CCA	AGC	CTA	TGC	CTA	CAG	CAT
	TCT	CCG	GGC	CGT	CAT	GGC	CGT	ATT	GGT	TCG	GAT	ACG	GAT	GTC	GTA
3286	CCA	GGG	TGA	CGG	TGC	CGA	GGA	TGA	CGA	TGA	CCG	CAT	TGT	TAG	ATT
	GGT	CCC	ACT	GCC	ACG	GCT	CCT	ACT	GCT	ACT	CGC	GTA	ACA	ATC	TAA

Fig. 10b (Con't)

3331	TCA	TAC	ACG	GTG	CCT	GAC	TGC	GTT	AGC	AAT	TTA	ACT	GTG	ATA	AAC
	AGT	ATG	TGC	CAC	GGA	CTG	ACG	CAA	TCG	TTA	AAT	TGA	CAC	TAT	TTG
3376	TAC	CGC	ATT	AAA	GCT	TAT	CGA	TGA	TAA	GCT	GTC	AAA	CAT	GAG	AAT
	ATG	GCG	TAA	TTT	CGA	ATA	GCT	ACT	ATT	CGA	CAG	TTT	GTA	CTC	TTA
3421	TAA														
	ATT														

Total number of bases is: 3423.

DNA sequence composition: 839 A; 915 C; 967 G; 702 T;

Sequence name: NIPS0060.

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fig.11a

Fig. 11b (Con't.)

361	GCC	TCT	AGA	GTC	GAC	CGG	CAT	GCA	AGC	TTA	AGT	AAG	TAA	GCC	GCC
	CGG	AGA	TCT	CAG	CTG	GCC	GTA	CGT	TCG	AAT	TCA	TTC	ATT	CGG	CGG
406	AGT	TCC	GCT	GGC	GGC	ATT	TTN	NTT	GAT	GCC	CAA	GCT	TGG	CTG	TTT
	TCA	AGG	CGA	CCG	CCG	TAA	AAN	NAA	CTA	CGG	GTT	CGA	ACC	GAC	AAA
451	TGG	CGG	ATG	AGA	GAA	GAT	TTT	CAG	CCT	GAT	ACA	GAT	TAA	ATC	AGA
	ACC	GCC	TAC	TCT	CTT	CTA	AAA	GTC	GGA	CTA	TGT	CTA	ATT	TAG	TCT
496	ACG	CAG	AAG	CGG	TCT	GAT	AAA	ACA	GAA	TTT	GCC	TGG	CGG	CAG	TAG
	TGC	GTC	TTC	GCC	AGA	CTA	TTT	TGT	CTT	AAA	CGG	ACC	GCC	GTC	ATC
541	CGC	GGT	GGT	CCC	ACC	TGA	CCC	CAT	GCC	GAA	CTC	AGA	AGT	GAA	ACG
	GCG	CCA	CCA	GGG	TGG	ACT	GGG	GTA	CGG	CTT	GAG	TCT	TCA	CTT	TGC
586	CCG	TAG	CGC	CGA	TGG	TAG	TGT	GGG	GTC	TCC	CCA	TGC	GAG	AGT	AGG
	GGC	ATC	GCG	GCT	ACC	ATC	ACA	CCC	CAG	AGG	GGT	ACG	CTC	TCA	TCC
631	GAA	CTG	CCA	GGC	ATC	AAA	TAA	AAC	GAA	AGG	CTC	AGT	CGA	AAG	ACT
	CTT	GAC	GGT	CCG	TAG	TTT	ATT	TTG	CTT	TCC	GAG	TCA	GCT	TTC	TGA
676	GGG	CCT	TTC	GTT	TTA	TCT	GTT	GTT	TGT	CGG	TGA	ACG	CTC	TCC	TGA
	CCC	GGA	AAG	CAA	AAT	AGA	CAA	CAA	ACA	GCC	ACT	TGC	GAG	AGG	ACT
721	GTA	GGA	CAA	ATC	CGC	CGG	GAG	CGG	ATT	TGA	ACG	TTG	CGA	AGC	AAC
	CAT	CCT	GTT	TAG	GCG	GCC	CTC	GCC	TAA	ACT	TGC	AAC	GCT	TCG	TTG

Fig. 11b (Con't.)

766	GGC CCG	CCG GGC	GAG CTC	GAG CCA	GGT CCA	GGC CCG	GGG CCC	CAG GTC	CAG CTC	GAC CTG	GCC CGG	CGC GCG	CAT GTA	AAA TTT	CTG GAC	CCA GGT	GCC CCG
811	ATC TAG	AAA TTT	TTA AAT	AGC TCG	AGA TCT	AGG TCC	AGG TCC	CCA GGT	CCA GGT	TCC AGG	TGA ACT	CGG GCC	ATG TAC	GCC CGG	TTT AAA	TTG AAC	CGT GCA
856	TTC AAG	TAC ATG	AAA TTT	CTC GAG	CTC GAG	TGT AAA	TGT ACA	TTA AAT	TTA AAT	TTT AAA	TTC AAG	TAA ATT	ATA TAT	CAT GTA	TCA AGT	AAT TTA	ATG TAC
901	TAT ATA	CCG GGC	CTC GAG	ATG TAC	AGA TCT	CAA TCT	CAA TCT	TAA ATT	TAA ATT	CCC GGG	TGA ACT	TAA ATT	ATG TAC	CTT GAA	CAA GTT	TAA ATT	TAA ATT
946	AAG TTC	GAT CTA	CTA GAT	GGT CCA	GAT CTA	GAT CTA	GAT CTA	CCT GGA	CCT GGA	TTT AAA	TGA ACT	TAA ATT	TCT AGA	CAT GTA	GAC CTG	CAA GTT	AAT TTA
991	CCC GGG	TTA AAT	ACG TGC	TGA ACT	GTI CAA	TTC AAG	TTC AAG	GTI CAA	GTI CAA	CCA GGT	CTG GAC	AGC TCG	GTC CAG	AGA TCT	CCC GGG	CGT GCA	AGA TCT
1036	AAA TTT	GAT CTA	CAA GTT	AGG TCC	ATC TAG	TTC AAG	TTC AAG	TTG AAC	TTG AAC	AGA TCT	TCC AGG	TTT AAA	TTT AAA	TCT AGA	GCG CGC	CGT GCA	AAT TTA

Fig. 11b (Con't)

1081	CTG	CTG	CTT	GCA	AAC	AAA	ACC	ACC	GCT	ACC	AGC	GGT	GGT	TTG
	GAC	GAC	GAA	CGT	TTG	TTT	TTT	TGG	CGA	TGG	TCG	CCA	CCA	AAC
1126	TTT	GCC	GGA	TCA	AGA	GCT	ACC	AAC	TCT	TCT	GAA	GGT	AAC	TGG
	AAA	CGG	CCT	AGT	TCT	CGA	TGG	TTG	AGA	AGG	CTT	CCA	TTG	ACC
1171	CTT	CAG	CAG	AGC	GCA	GAT	ACC	AAA	TAC	TGT	TCT	AGT	GTA	GCC
	GAA	GTC	GTC	TCG	CGT	CTA	TGG	TTT	ATG	ACA	AGA	TCA	CAT	CGG
1216	GTA	GTT	AGG	CCA	CCA	CTT	CAA	GAA	CTC	TGT	AGC	ACC	TAC	ATA
	CAT	CAA	TCC	GGT	GGT	GAA	GTT	CTT	GAG	ACA	TCG	TGG	ATG	TAT
1261	CCT	CGC	TCT	GCT	AAT	CCT	GTT	ACC	AGT	GGC	TGC	CAG	TGG	CGA
	GGA	GCG	AGA	CGA	TTA	GGA	CAA	TGG	TCA	CCG	ACG	GTC	ACC	GCT
1306	TAA	GTC	GTG	TCT	TAC	CGG	GTT	GGA	CTC	AAG	ACG	ATA	GTT	GGA
	ATT	CAG	CAC	AGA	ATG	GCC	CAA	CCT	GAG	TTC	TGC	TAT	CAA	TGG
1351	TAA	GGC	GCA	GCG	GTC	GGG	CTG	AAC	GGG	TTC	GTG	CAC	ACA	GCC
	ATT	CCG	CGT	CGC	CAG	CCC	GAC	TTG	CCC	AAG	CAC	GTG	TGT	CGG

Fig. 11b (Con't)

1396	CAG	CTT	GGA	GCG	AAC	GAC	CTA	CAC	CGA	ACT	GAG	ATA	CCT	ACA	GCG
	GTC	GAA	CCT	CGC	TTG	CTG	GAT	GTG	GCT	TGA	CTC	TAT	GGA	TGT	CGC
1441	TGA	GCA	TTG	AGA	AAG	CGC	CAC	GCT	TCC	CGA	AGG	GAG	AAA	GGC	GGA
	ACT	CGT	AAC	TCT	TTC	GCG	GTG	CGA	AGG	GCT	TCC	CTC	TTT	CCG	CCT
1486	CAG	GTA	TCC	GGT	AAG	CGG	CAG	GGT	CGG	AAC	AGG	AGA	GCG	CAC	GAG
	GTC	CAT	AGG	CCA	TTC	GCC	GTC	CCA	GCC	TTG	TCC	TCT	CGC	GTG	CTC
1531	GGA	GCT	TCC	AGG	GGG	AAA	CGC	CTG	GTA	TCT	TTA	TAG	TCC	TGT	CGG
	CCT	CGA	AGG	TCC	CCC	TTT	GCG	GAC	CAT	AGA	AAT	ATC	AGG	ACA	GCC
1576	GTT	TCG	CCA	CCT	CTG	ACT	TGA	GCG	TCG	ATT	TTT	GTG	ATG	CTC	GTC
	CAA	AGC	GGT	GGA	GAC	TGA	ACT	CGC	AGC	TAA	AAA	CAC	TAC	GAG	CAG

Fig. 11b (Con't)

1621	AGG	GGG	GCG	GAG	CCT	ATG	GAA	AAA	CGC	CAG	CAA	CGC	GGC	CTT	TTT
	TCC	CCC	CGC	CTC	GGA	TAC	CTT	TTT	GCG	GTC	GTT	GCG	CCG	GAA	AAA
1666	ACG	GTT	CCT	GGC	CTT	TTG	CTG	GCC	TTT	TGC	TCA	CAT	GTT	CTT	TCC
	TGC	CAA	GGA	CCG	GAA	AAC	GAC	CGG	AAA	ACG	AGT	GTA	CAA	GAA	AGG
1711	TGC	GTT	ATC	CCC	TGA	TTC	TGT	GGA	TAA	CCG	TAT	TAC	CGC	CTT	TGA
	ACG	CAA	TAG	GGG	ACT	AAG	ACA	CCT	ATT	GGC	ATA	ATG	GCG	GAA	ACT
1756	GTG	AGC	TGA	TAC	CGC	TCG	CCG	CAG	CCG	AAC	GAC	CGA	GCG	CAG	CGA
	CAC	TCG	ACT	ATG	GCG	AGC	GGC	GTC	GGC	TTG	CTG	GCT	CGC	GTC	GCT
1801	GTC	AGT	GAG	CGA	GGA	AGC	GGA	AGA	GCG	CTG	ACT	TCC	GCG	TTT	CCA
	CAG	TCA	CTC	GCT	CCT	TCG	CCT	TCT	CGC	GAC	TGA	AGG	CGC	AAA	GGT
1846	GAC	TTT	ACG	AAA	CAC	GGA	AAC	CGA	AGA	CCA	TTC	ATG	TTG	TTG	CTC
	CTG	AAA	TGC	TTT	GTG	CCT	TTG	GCT	TCT	GGT	AAG	TAC	AAC	AAC	GAG
1891	AGG	TCG	CAG	ACG	TTT	TGC	AGC	AGC	AGT	CGC	TTC	ACG	TTC	GCT	CGC
	TCC	AGC	GTC	TGC	AAA	ACG	TCG	TCG	TCA	GCG	AAG	TGC	AAG	CGA	GCG

Fig. 11b (Con't)

1936	GTA	TCG	GTG	ATT	CAT	TCT	GCT	AAC	CAG	TAA	GGC	AAC	CCC	GCC	AGC
	CAT	AGC	CAC	TAA	GTA	AGA	CGA	TTG	GTC	ATT	CCG	TTG	GGG	CGG	TCG
1981	CTA	GCC	GGG	TCC	TCA	ACG	ACA	GGA	GCA	CGA	TCA	TGC	GCA	CCC	GTG
	GAT	CGG	CCC	AGG	AGT	TGC	TGT	CCT	CGT	GCT	AGT	ACG	CGT	GGG	CAC
2026	GCC	AGG	ACC	CAA	CGC	TGC	CCG	AGA	TGC	GCC	GCG	TGC	GGC	TGC	TGG
	CGG	TCC	TGG	GTT	GCG	ACG	GGC	TCT	ACG	CGG	CGC	ACG	CCG	ACG	ACC
2071	AGA	TGG	CGG	ACG	CGA	TGG	ATA	TGT	TCT	GCC	AAG	GGT	TGG	TTT	GCG
	TCT	ACC	GCC	TGC	GCT	ACC	TAT	ACA	AGA	CGG	TTC	CCA	ACC	AAA	CGC
2116	CAT	TCA	CAG	TTC	TCC	GCA	AGA	ATT	GAT	TGG	CTC	CAA	TTC	TTG	GAG
	GTA	AGT	GTC	AAG	AGG	CGT	TCT	TAA	CTA	ACC	GAG	GTT	AAG	AAC	CTC
2161	TGG	TGA	ATC	CGT	TAG	CGA	GGT	GCC	GCC	GGC	TTC	CAT	TCA	GGT	CGA
	ACC	ACT	TAG	GCA	ATC	GCT	CCA	CGG	CGG	CCG	AAG	GTA	AGT	CCA	GCT
2206	GGT	GGC	CCG	GCT	CCA	TGC	ACC	GCG	ACG	CAA	CGC	GGG	GAG	GCA	GAC
	CCA	CCG	GGC	CGA	GGT	ACG	TGG	CGC	TGC	GTT	GCG	CCC	CTC	CGT	CTG

Fig. 11b (Con't)

2251	AAG	GTA	TAG	GGC	GGC	GCC	TAC	AAT	CCA	TGC	CAA	CCC	GTT	CCA	TGT
	TTC	CAT	ATC	CCG	CCG	CGG	ATG	TTA	GGT	ACG	GTT	GGG	CAA	GGT	ACA
2296	GCT	CGC	CGA	GGC	GGC	ATA	AAT	CGC	CGT	GAC	GAT	CAG	CGG	TCC	AGT
	CGA	GCG	GCT	CCG	CCG	TAT	TTA	GCG	GCA	CTG	CTA	GTC	GCC	AGG	TCA
2341	GAT	CGA	AGT	TAG	GCT	GGT	AAG	AGC	CGC	GAG	CGA	TCC	TTG	AAG	CTG
	CTA	GCT	TCA	ATC	CGA	CCA	TTC	TCG	GCG	CTC	GCT	AGG	AAC	TTC	GAC
2386	TCC	CTG	ATG	GTC	GTC	ATC	TAC	CTG	CCT	GGA	CAG	CAT	GGC	CTG	CAA
	AGG	GAC	TAC	CAG	CAG	TAG	ATG	GAC	GGA	CCT	GTC	GTA	CCG	GAC	GTT
2431	CGC	GGG	CAT	CCC	GAT	GCC	GCC	GGA	AGC	GAG	AAG	AAT	CAT	AAT	GGG
	GCG	CCC	GTA	GGG	CTA	CGG	CGG	CCT	TCG	CTC	TTC	TTA	GTA	TTA	CCC

Fig. 11b (Con't)

2476	GAA	GGC	CAT	CCA	GCC	TCG	CGT	CGC	GAA	CGC	CAG	CAA	GAC	GTA	GCC
	CTT	CCG	GTA	GGT	CGG	AGC	GCA	GGC	CTT	GCG	GTC	GTT	CTG	CAT	CGG
2521	CAG	CGC	GTC	GGC	CGC	CAT	GCC	GGC	GAT	AAT	GGC	CTG	CTT	CTC	GCC
	GTC	GGC	CAG	CCG	GGC	GTA	CGG	CCG	CTA	TTA	CCG	GAC	GAA	GAG	CGG
2566	GAA	ACG	TTT	GGT	GGC	GGG	ACC	AGT	CAC	GAA	GGC	TTG	AGC	GAG	GGC
	CTT	TGC	AAA	CCA	CCG	CCC	TGG	TCA	CTG	CTT	CCG	AAC	TCG	CTC	CCG
2611	GTG	CAA	GAT	TCC	GAA	TAC	CGC	AAG	CGA	CAG	GCC	GAT	CAT	CGT	CGC
	CAC	GTT	CTA	AGG	CTT	ATG	GCG	TTC	GCT	GTC	CGG	CTA	GTA	GCA	GCG
2656	GCT	CCA	GCG	AAA	GCG	GTC	CTC	GCC	GAA	AAT	GAC	CCA	GAG	CGC	TGC
	CGA	GGT	CGC	TTT	CGC	CAG	GAG	CGG	CTT	TTA	CTG	GGT	CTC	GCG	ACG
2701	CGG	CAC	CTG	TCC	TAC	GAG	TTG	CAT	GAT	AAA	GAA	GAC	AGT	CAT	AAG
	GCC	GTG	GAC	AGG	ATG	CTC	AAC	GTA	CTA	TTT	CTT	CTG	TCA	GTA	TTC
2746	TGC	GGC	GAC	GAT	AGT	CAT	GCC	CCG	CGC	CCA	CCG	GAA	GGA	GCT	GAC
	ACG	CCG	CTG	CTA	TCA	GTA	CGG	GGC	GCG	GGT	GCG	CTT	CCT	CGA	CTG

Fig. 11b (Con't)

2791	TGG	GTT	GAA	GGC	TCT	CAA	GGG	CAT	CGG	TCG	ACG	CTC	TCC	CTT	ATG
	ACC	CAA	CTT	CCG	AGA	GTT	CCC	GTA	GCC	AGC	TGC	GAG	AGG	GAA	TAC
2836	CGA	CTC	CTG	CAT	TAG	GAA	GCA	GCC	CAG	TAG	TAG	GTT	GAG	GCC	GTT
	GCT	GAG	GAC	GTA	ATC	CTT	CGT	CGG	GTC	ATC	ATC	CAA	CTC	CGG	CAA
2881	GAG	CAC	CGC	CGC	CGC	AAG	GAA	TGG	TGC	ATG	CAA	GGA	GAT	GGC	GCC
	CTC	GTG	GCG	GCG	GCG	TTC	CTT	ACC	ACG	TAC	GTT	CCT	CTA	CCG	CGG
2926	CAA	CAG	TCC	CCC	GGC	CAC	GGG	GCC	TGC	CAC	CAT	ACC	CAC	GCC	GAA
	GTT	GTC	AGG	GGG	CCG	GTG	CCC	CGG	ACG	GTG	GTA	TGG	GTG	CGG	CTT
2971	ACA	AGC	GCT	CAT	GAG	CCC	GAA	GTG	GCG	AGC	CCG	ATC	TTC	CCC	ATC
	TGT	TCG	CGA	GTA	CTC	GGG	CTT	CAC	CGC	TCG	GGC	TAG	AAG	GGG	TAG
3016	GGT	GAT	GTC	GGC	GAT	ATA	GGC	GCC	AGC	AAC	CGC	ACC	TGT	GGC	GCC
	CCA	CTA	CAG	CCG	CTA	TAT	CCG	CGG	TCG	TTG	GCG	TGG	ACA	CCG	CGG
3061	GGT	GAT	GCC	GGC	CAC	GAT	GCG	TCC	GGC	GTA	GAG	GAT	CCA	CAG	GAC
	CCA	CTA	CGG	CCG	GTG	CTA	CGC	AGG	CCG	CAT	CTC	CTA	GGT	GTC	CTG

Fig. 11b (Con't)

3106	GGG	TGT	GGT	CGC	CAT	GAT	CGC	GTA	GTC	GAT	AGT	GGC	TCC	AAG	TAG
	CCC	ACA	CCA	GCG	GTA	CTA	GCG	CAT	CAG	CTA	TCA	CCG	AGG	TTC	ATC
3151	CGA	AGC	GAG	CAG	GAC	TGG	GCG	GCG	GCC	AAA	GCG	GTC	GGA	CAG	TGC
	GCT	TCG	CTC	GTC	CTG	ACC	CGC	CGC	CGG	TTT	CGC	CAG	CCT	GTC	ACG
3196	TCC	GAG	AAC	GGG	TGC	GCA	TAG	AAA	TTG	CAT	CAA	CGC	ATA	TAG	CGC
	AGG	CTC	TTG	CCC	ACG	CGT	ATC	TTT	AAC	GTA	GTT	GCG	TAT	ATC	GCG
3241	TAG	CAG	CAC	GCC	ATA	GTG	ACT	GGC	GAT	GCT	GTC	GGA	ATG	GAC	GAT
	ATC	GTC	GTG	CGG	TAT	CAC	TGA	CCG	CTA	CGA	CAG	CCT	TAC	CTG	CTA
3286	ATC	CCG	CAA	GAG	GCC	CGG	CAG	TAC	CGG	CAT	AAC	CAA	GCC	TAT	GCC
	TAG	GGC	GTT	CTC	CGG	GCC	GTC	ATG	GCC	GTA	TTG	GTT	CGG	ATA	CGG

Fig. 11b (Con't)

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3331 TAC AGC ATC CAG GGT GAC GGT GCC GAG GAT GAG CGC ATT
    ATG TCG TAG GTC CCA CTG CCA CGG CTC CTA CTG CTC GCG TAA

3376 GTT AGA TTT CAT ACA CGG TGC CTG ACT GCG TTA GCA ATT TAA CTG
    CAA TCT AAA GTA TGT GCC ACG GAC TGA CGC AAT CGT TAA ATT GAC

3421 TGA TAA ACT ACC GCA TTA AAG CTT ATC GAT AAG CTG TCA AAC
    ACT ATT TGA TGG CGT AAT TTC GAA TAG CTA TTC GAC AGT TTG

3466 ATG AGA ATT
    TAC TCT TAA

```

Total number of bases is: 3474.

DNA sequence composition: 845 A; 933 C; 978 G; 716 T;

2 OTHER;

Sequence name: NPMTNFMMPH.

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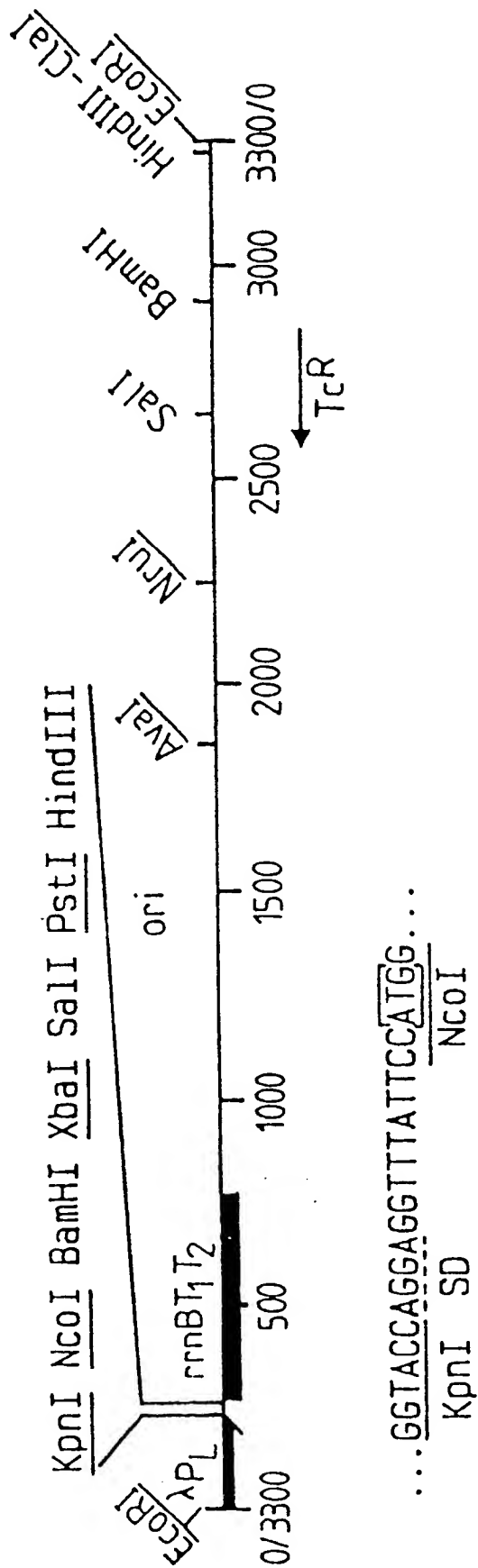


fig.12a

From: pIG2

Fig. 12b

	3	9	15	21	27	33	39	45
	1	1	1	1	1	1	1	1
	TTC	CGG	GGA	TCT	CTC	ACC	TAC	CAA
	AAG	GCC	CCT	AGA	GAG	TGG	ATG	GTT
	46	4	10	16	22	28	34	40
	TAA	ATT	CAT	ATA	AAA	AAC	ATA	CAG
	ATT	TAA	GTA	TAT	TTT	TTG	TAT	GTC
	91	11	17	23	29	35	41	47
	TTA	TCT	CTG	GCG	GTG	TTG	ACA	TAA
	AAT	AGA	GAC	CGC	CAC	AAC	TGT	ATT
	136	12	18	24	30	36	42	48
	AGC	ACA	TCA	GCA	GGA	CGC	ACT	GAC
	TCG	TGT	AGT	CGT	CCT	GCG	TGA	CTG
	181	13	19	25	31	37	43	49
	AAA	AAT	TAA	GCC	CTG	AAG	AAG	GGC
	TTT	TTA	ATT	CGG	GAC	TTC	TTC	CCG
	226	14	20	26	32	38	44	50
	TAT	TCC	ATG	GGG	GGG	ATC	CTC	TAG
	ATA	AGG	TAC	CCC	CCC	TAG	GAG	ATC
	271	15	21	27	33	39	45	51
	TGG	CTG	TTT	TGG	CGG	ATG	AGA	GAA
	ACC	GAC	AAA	ACC	GCC	TAC	TCT	CTT
	316	16	22	28	34	40	46	52
	TAA	ATC	AGA	ACG	CAG	AAG	CGG	TCT
	ATT	TAG	TCT	TGC	GTC	TTC	GCC	AGA

Fig. 12b (con't)

361	CGG	CAG	TAG	CGC	GGT	GGT	CCC	ACC	TGA	CCC	CAT	GCC	GAA	CTC	AGA
	GCC	GTC	ATC	GCG	CCA	CCA	GGG	TGG	ACT	GGG	GTA	CGG	CTT	GAG	TCT
406	AGT	GAA	ACG	CCG	TAG	CGC	CGA	TGG	TAG	TGT	GGG	GTC	TCC	CCA	TGC
	TCA	CTT	TGC	GCG	ATC	GCG	GCT	ACC	ATC	ACA	CCC	CAG	AGG	GGT	ACG
451	GAG	AGT	AGG	GAA	CTG	CCA	GCC	ATC	AAA	TAA	AAC	GAA	AGG	CTC	AGT
	CTC	TCA	TCC	CTT	GAC	GGT	CCG	TAG	TTT	ATT	TTG	CTT	TCC	GAG	TCA
496	CGA	AAG	ACT	GGG	CCT	TTC	GTT	TTA	TCT	GTT	GTT	TGT	CGG	TGA	ACG
	GCT	TTC	TGA	CCC	GGA	AAG	CAA	AAT	AGA	CAA	CAA	ACA	GCC	ACT	TGC
541	CTC	TCC	TGA	GTA	GGA	CAA	ATC	CGC	CGG	GAG	CGG	ATT	TGA	ACG	TTG
	GAG	AGG	ACT	CAT	CCT	GTT	TAG	GCG	GCC	CTC	GCC	TAA	ACT	TGC	AAC
586	CGA	AGC	AAC	GGC	CCG	GAG	GGT	GGC	GGG	CAG	GAC	GCC	CGC	CAT	AAA
	GCT	TCG	TTG	CCG	GGC	CTC	CCA	CCG	CCC	GTC	CTG	CGG	GCG	GTA	TTT
631	CTG	CCA	GGC	ATC	AAA	TTA	AGC	AGA	AGG	CCA	TCC	TGA	CGG	ATG	GCC
	GAC	GGT	CCG	TAG	TTT	AAT	TCG	TCT	TCC	GGT	AGG	ACT	GCC	TAC	CGG
676	TTT	TTG	CGT	TTC	TAC	AAA	CTC	TTT	TGT	TTA	TTT	TTC	TAA	ATA	CAT
	AAA	AAC	GCA	AAG	ATG	TTT	GAG	AAA	ACA	AAT	AAA	AAG	ATT	TAT	GTA
721	TCA	AAT	ATG	TAT	CCG	CTC	ATG	AGA	CAA	TAA	CCC	TGA	TAA	ATG	CTT
	AGT	TTA	TAC	ATA	GCG	GAG	TAC	TCT	GTT	ATT	GGG	ACT	ATT	TAC	GAA

Fig. 12b (Con't)

766	CAA	TAA	TAA	AAG	GAT	CTA	GGT	GAA	GAT	CCT	TTT	TGA	TAA	TCT	CAT
	GTT	ATT	ATT	TTC	CTA	GAT	CCA	CTT	CTA	GGA	AAA	ACT	ATT	AGA	GTA
811	GAC	CAA	AAT	CCC	TTA	ACG	TGA	GTT	TTC	GTT	CCA	CTG	AGC	GTC	AGA
	CTG	GTT	TTA	GGG	AAT	TGC	ACT	CAA	AAG	CAA	GGT	GAC	TCG	CAG	TCT
856	CCC	CGT	AGA	AAA	GAT	CAA	AGG	ATC	TTC	TTG	AGA	TCC	TTT	TTT	TCT
	GGG	GCA	TCT	TTT	CTA	GTT	TCC	TAG	AAG	AAC	TCT	AGG	AAA	AAA	AGA
901	GCG	CGT	AAT	CTG	CTG	CTT	GCA	AAC	AAA	AAA	ACC	ACC	GCT	ACC	AGC
	CGC	GCA	TTA	GAC	GAC	GAA	CGT	TTG	TTT	TTT	TGG	TGG	CGA	TGG	TCG
946	GGT	GGT	TTG	TTT	GCC	GGA	TCA	AGA	GCT	ACC	AAC	TCT	TTT	TCC	GAA
	CCA	CCA	AAC	AAA	CGG	CCT	AGT	TCT	CGA	TGG	TTG	AGA	AAA	AGG	CTT
991	GGT	AAC	TGG	CTT	CAG	CAG	AGC	GCA	GAT	ACC	AAA	TAC	TGT	CCT	TCT
	CCA	TTG	ACC	GAA	GTC	GTC	TCG	CGT	CTA	TGG	TTT	ATG	ACA	GGA	AGA
1036	AGT	GTA	GCC	GTA	GTT	AGG	CCA	CCA	CTT	CAA	GAA	CTC	TGT	AGC	ACC
	TCA	CAT	CGG	CAT	CAA	TCC	GGT	GGT	GAA	GTT	CTT	GAG	ACA	TCG	TGG

Fig. 12b (Con't)

1081	GCC	TAC	ATA	CCT	CGC	TCT	GCT	AAT	CCT	GTT	ACC	AGT	GGC	TGC	TGC
	CGG	ATG	TAT	GGA	GCG	AGA	CGA	TTA	GGA	CAA	TGG	TCA	CCG	ACG	ACG
1126	CAG	TGG	CGA	TAA	GTC	GTG	TCT	TAC	CGG	GTT	GGA	CTC	AAG	ACG	ATA
	GTC	ACC	GCT	ATT	CAG	CAC	AGA	ATG	GCC	CAA	CCT	GAG	TTC	TGC	TAT
1171	GTT	ACC	GGA	TAA	GGC	GCA	GCG	GTC	GGG	CTG	AAC	GGG	GGG	TTC	GTG
	CAA	TGG	CCT	ATT	CCG	CGT	CGC	CAG	CCC	GAC	TTG	CCC	CCC	AAG	CAC
1216	CAC	ACA	GCC	CAG	CTT	GGA	GCG	AAC	GAC	CTA	CAC	CGA	ACT	GAG	ATA
	GTG	TGT	CGG	GTC	GAA	CCT	CGC	TTG	CTG	GAT	GTG	GCT	TGA	CTC	TAT
1261	CCT	ACA	GCG	TGA	GCA	TTG	AGA	AAG	CGC	CAC	GCT	TCC	CGA	AGG	GAG
	GGA	TGT	CGC	ACT	CGT	AAC	TCT	TTC	GCG	GTG	CGA	AGG	GCT	TCC	CTC
1306	AAA	GGC	GGA	CAG	GTA	TCC	GGT	AAG	CGG	CAG	GGT	CGG	AAC	AGG	AGA
	TTT	CCG	CCT	GTC	CAT	AGG	CCA	TTC	GCC	GTC	CCA	GCC	TTG	TCC	TCT
1351	GCG	CAC	GAG	GGA	GCT	TCC	AGG	GGG	AAA	CGC	CTG	GTA	TCT	TTA	TAG
	CGC	GTG	CTC	CCT	CGA	AGG	TCC	CCC	TTT	GCG	GAC	CAT	AGA	AAT	ATC

Fig. 12b (Con't)

1396	TCC	TGT	CGG	GTT	TCG	CCA	CCT	CTG	ACT	TGA	GCG	TCG	ATT	TTT	GTG
	AGG	ACA	GCC	CAA	AGC	GGT	GGA	GAC	TGA	ACT	CGC	AGC	TAA	AAA	CAC
1441	ATG	CTC	GTC	AGG	GGG	GCG	GAG	CCT	ATG	GAA	AAA	CGC	CAG	CAA	CGC
	TAC	GAG	CAG	TCC	CCC	CGC	CTC	GGA	TAC	CTT	TTT	GCG	GTC	GTT	GCG
1486	GGC	CTT	TTT	ACG	GTT	CCT	GGC	CTT	TTG	CTG	GCC	TTT	TGC	TCA	CAT
	CCG	GAA	AAA	TGC	CAA	GGA	CCG	GAA	AAC	GAC	CGG	AAA	ACG	AGT	GTA
1531	GTT	CTT	TCC	TGC	GTT	ATC	CCC	TGA	TTC	TGT	GGA	TAA	CCG	TAT	TAC
	CAA	GAA	AGG	ACG	CAA	TAG	GGG	ACT	AAG	ACA	CCT	ATT	GGC	ATA	ATG
1576	CGC	CTT	TGA	GTG	AGC	TGA	TAC	CGC	TCG	CCG	CAG	CCG	AAC	GAC	CGA
	GCG	GAA	ACT	CAC	TCG	ACT	ATG	GCG	AGC	GGC	GTC	GGC	TTG	CTG	GCT

Fig. 12b (Con't)

1621	GCG	CAG	CGA	GTC	AGT	GAG	CGA	GGA	AGC	GGA	AGA	GCG	CTG	ACT	TCC
	CGC	GTC	GCT	CAG	TCA	CTC	GCT	CCT	TCG	CCT	TCT	CGC	GAC	TGA	AGG
1666	GCG	TTT	CCA	GAC	TTT	ACG	AAA	CAC	GGA	AAC	CGA	AGA	CCA	TTC	ATG
	CGC	AAA	GGT	CTG	AAA	TGC	TTT	GTG	CCT	TTG	GCT	TCT	GGT	AAG	TAC
1711	TTG	TTG	CTC	AGG	TCG	CAG	ACG	TTT	TGC	AGC	AGC	AGT	CGC	TTC	ACG
	AAC	AAC	GAG	TCC	AGC	GTC	TGC	AAA	ACG	TCG	TCG	TCA	GCG	AAG	TGC
1756	TTC	GCT	CGC	GTA	TCG	GTG	ATT	CAT	TCT	GCT	AAC	CAG	TAA	GGC	AAC
	AAG	CGA	GCG	CAT	AGC	CAC	TAA	GTA	AGA	CGA	TTG	GTC	ATT	CCG	TTG
1801	CCC	GCC	AGC	CTA	GCC	GGG	TCC	TCA	ACG	ACA	GGA	GCA	CGA	TCA	TGC
	GGG	CGG	TCG	GAT	CGG	CCC	AGG	AGT	TGC	TGT	CCT	CGT	GCT	AGT	ACG
1846	GCA	CCC	GTG	GCC	AGG	ACC	CAA	CGC	TGC	CCG	AGA	TGC	GCC	GCG	TGC
	CGT	GGG	CAC	CGG	TCC	TGG	GTT	GCG	ACG	GGC	TCT	ACG	CGG	CGC	ACG
1891	GGC	TGC	TGG	AGA	TGG	CGG	ACG	CGA	TGG	ATA	TGT	TCT	GCC	AAG	GGT
	CCG	ACG	ACC	TCT	ACC	GCC	TGC	GCT	ACC	TAT	ACA	AGA	CGG	TTC	CCA

Fig. 12b (Con't.)

1936	TGG ACC	TTT AAA	GCG CGC	CAT GTA	TCA AGT	CAG GTC	TTC AAG	TCC AGG	GCA CGT	AGA TCT	ATT TAA	GAT CTA	TGG ACC	CTC GAG	CAA GTT
1981	TTC AAG	TTG AAC	GAG CTC	TGG ACC	TGA ACT	ATC TAG	CGT GCA	TAG ATC	CGA GCT	GGT CCA	GCC CGG	GCC CGG	GGC CCG	TTC AAG	CAT GTA
2026	TCA AGT	GGT CCA	CGA GCT	GGT CCA	GGC CCG	CCG GGC	GCT CGA	CCA GGT	TGC ACG	ACC TGG	GCG CGC	ACG TGC	CAA GTT	CGC GCG	GGG CCC
2071	GAG CTC	GCA CGT	GAC CTG	AAG TTC	GTA CAT	TAG ATC	GGC CCG	GGC CCG	GCC CGG	TAC ATG	AAT TTA	CCA GGT	TGC ACG	CAA GTT	CCC GGG
2116	GTT CAA	CCA GGT	TGT ACA	GCT CGA	CGC GCG	CGA GCT	GGC CCG	GGC CCG	ATA TAT	AAT TTA	CGC GCG	CGT GCA	GAC CTG	GAT CTA	CAG GTC
2161	CGG GCC	TCC AGG	AGT TCA	GAT CTA	CGA GCT	AGT TCA	TAG ATC	GCT CGA	GGT CCA	AAG TTC	AGC TCG	CGC GCG	GAG CTC	CGA GCT	TCC AGG
2206	TTG AAC	AAG TTC	CTG GAC	TCC AGG	CTG GAC	ATG TAC	GTC CAG	GTC CAG	ATC TAG	TAC ATG	CTG GAC	CCT GGA	GGA CCT	CAG GTC	CAT GTA

Fig. 12b (Con't)

2251	GGC	CTG	CAA	CGC	GGG	CAT	CCC	GAT	GCC	GCC	GGA	AGC	GAG	AAG	AAT
	CCG	GAC	GTT	GCG	CCC	GTA	GGG	CTA	CGG	CGG	CCT	TCG	CTC	TTC	TTA
2296	CAT	AAT	GGG	GAA	GGC	CAT	CCA	GCC	TCG	CGT	CGC	GAA	CGC	CAG	CAA
	GTA	TTA	CCC	CTT	CCG	GTA	GGT	CGG	AGC	GCA	GCG	CTT	GCG	GTC	GTT
2341	GAC	GTA	GCC	CAG	CGC	GTC	GGC	CGC	CAT	GCC	GGC	GAT	AAT	GGC	CTG
	CTG	CAT	CGG	GTC	GGC	CAG	CCG	GCG	GTA	CGG	CCG	CTA	TTA	CCG	GAC
2386	CTT	CTC	GCC	GAA	ACG	TTT	GGT	GGC	GGG	ACC	AGT	GAC	GAA	GGC	TTG
	GAA	GAG	CGG	CTT	TGC	AAA	CCA	CCG	CCC	TGG	TCA	CTG	CTT	CCG	AAC
2431	AGC	GAG	GGC	GTG	CAA	GAT	TCC	GAA	TAC	CGC	AAG	CGA	CAG	GCC	GAT
	TCG	CTC	CCG	CAC	GTT	CTA	AGG	CTT	ATG	GCG	TTC	GCT	GTC	CGG	CTA

Fig. 12b (Con't)

2476	CAT GTA	CGT GCA	CGC GCG	GCT CGA	CCA GGT	CCG CGC	AAA TTT	GCG CGC	GTC CAG	CTC GAG	GCC CGG	GAA CTT	AAT TTA	GAC CTG	CCA GGT
2521	GAG CTC	CGC GCG	TGC ACG	CGG GCC	CAC GTG	CTG GAC	TCC AGG	TAC ATG	GAG CTC	TTG AAC	CAT GTA	GAT CTA	AAA TTT	GAA CTT	GAC CTG
2566	AGT TCA	CAT GTA	AAG TTC	TGC ACG	GGC CCG	GAC CTG	GAT CTA	AGT TCA	CAT GTA	GCC CGG	CCG GGC	CGC GCG	CCA GGT	CCG GGC	GAA CTT
2611	GGA CCT	GCT CGA	GAC CTG	TGG ACC	GTT CAA	GAA CTT	GGC CCG	TCT AGA	CAA GTT	GGG CCC	CAT GTA	CGG GCC	TCG AGC	ACG TGC	CTC GAG
2656	TCC AGG	CTT GAA	ATG TAC	CGA GCT	CTC GAG	CTG GAC	CAT GTA	TAG ATC	GAA CTT	GCA CGT	GCC CGG	CAG GTC	TAG ATC	TAG ATC	GTT CAA
2701	GAG CTC	GCC CGG	GTT CAA	GAG CTC	CAC GTG	CGC GCG	CGC GCG	CGC GCG	AAG TTC	GAA CTT	TGG ACC	TGC ACG	ATG TAC	CAA GTT	GGA CCT
2746	GAT CTA	GGC CCG	GCC CGG	CAA GTT	CAG GTC	TCC AGG	CCC GGG	GGC CCG	CAC GTG	GGG CCC	GCC CGG	TGC ACG	CAC GTG	CAT GTA	ACC TGG

Fig. 12b (Con't)

2791	CAC	GCC	GAA	ACA	AGC	GC'T	CAT	GAG	CCC	GAA	GTG	GCG	AGC	CCG	ATC
	GTG	CGG	CTT	TGT	TCG	CGA	GTA	CTC	GGG	CTT	CAC	CGC	TCG	GGC	TAG
2836	TTC	CCC	ATC	GGT	GAT	GTC	GGC	GAT	ATA	GGC	GCC	AGC	AAC	CGC	ACC
	AAG	GGG	TAG	CCA	CTA	CAG	CCG	CTA	TAT	CCG	CGG	TCG	TTG	GGC	TGG
2881	TGT	GGC	GCC	GGT	GAT	GCC	GGC	CAC	GAT	GCG	TCC	GGC	GTA	GAG	GAT
	ACA	CCG	CGG	CCA	CTA	CGG	CCG	GTG	CTA	CGC	AGG	CCG	CAT	CTC	CTA
2926	CCA	CAG	GAC	GGG	TGT	GGT	CGC	CAT	GAT	CGC	GTA	GTC	GAT	AGT	GGC
	GGT	GTC	CTG	CCC	ACA	CCA	GCG	GTA	CTA	GCG	CAT	CAG	CTA	TCA	CCG
2971	TCC	AAG	TAG	CGA	AGC	GAG	CAG	GAC	TGG	GCG	GCG	GCC	AAA	GCG	GTC
	AGG	TTC	ATC	GCT	TCG	CTC	GTC	CTG	ACC	CGC	CGC	CGG	TTT	CGC	CAG
3016	GGA	CAG	TGC	TCC	GAG	AAC	GGG	TGC	GCA	TAG	AAA	TTG	CAT	CAA	CGC
	CCT	GTC	ACG	AGG	CTC	TTG	CCC	ACG	CGT	ATC	TTT	AAC	GTA	GTT	GCG
3061	ATA	TAG	CGC	TAG	CAG	CAC	GCC	ATA	GTG	ACT	GGC	GAT	GCT	GTC	GGA
	TAT	ATC	GCG	ATC	GTC	GTG	CGG	TAT	CAC	TGA	CCG	CTA	CGA	CAG	CCT

Fig. 12b (Con't)

```

3106 ATG GAC GAT ATC CCG CAA GAG GCC CGG CAG TAC CGG CAT AAC CAA
      TAC CTG CTA TAG GGC GTT CTC CGG GCC GTC ATG GCC GTA TTG GTT

3151 GCC TAT GCC TAC AGC ATC CAG GGT GAC GGT GCC GAT GAC GAT
      CGG ATA CGG ATG TCG TAG GTC CCA CTG CCA CGG CTC CTA CTG CTA

3196 GAG CGC ATT GTT AGA TTT CAT ACA CGG TGC CTG ACT GCG TTA GCA
      CTC GCG TAA CAA TCT AAA GTA TGT GCC ACG GAC TGA CGC AAT CGT

3241 ATT TAA CTG TGA TAA ACT ACC GCA TTA AAG CTT ATC GAT AAG
      TAA ATT GAC ACT ATT TGA TGG CGT AAT TTC GAA TAG CTA CTA TTC

3286 CTG TCA AAC ATG AGA A
      GAC AGT TTG TAC TCT T

```

Total number of bases is: 3301.

DNA sequence composition: 797 A; 887 C; 936 G; 681 T;

Sequence name: NIPS0039.

=====

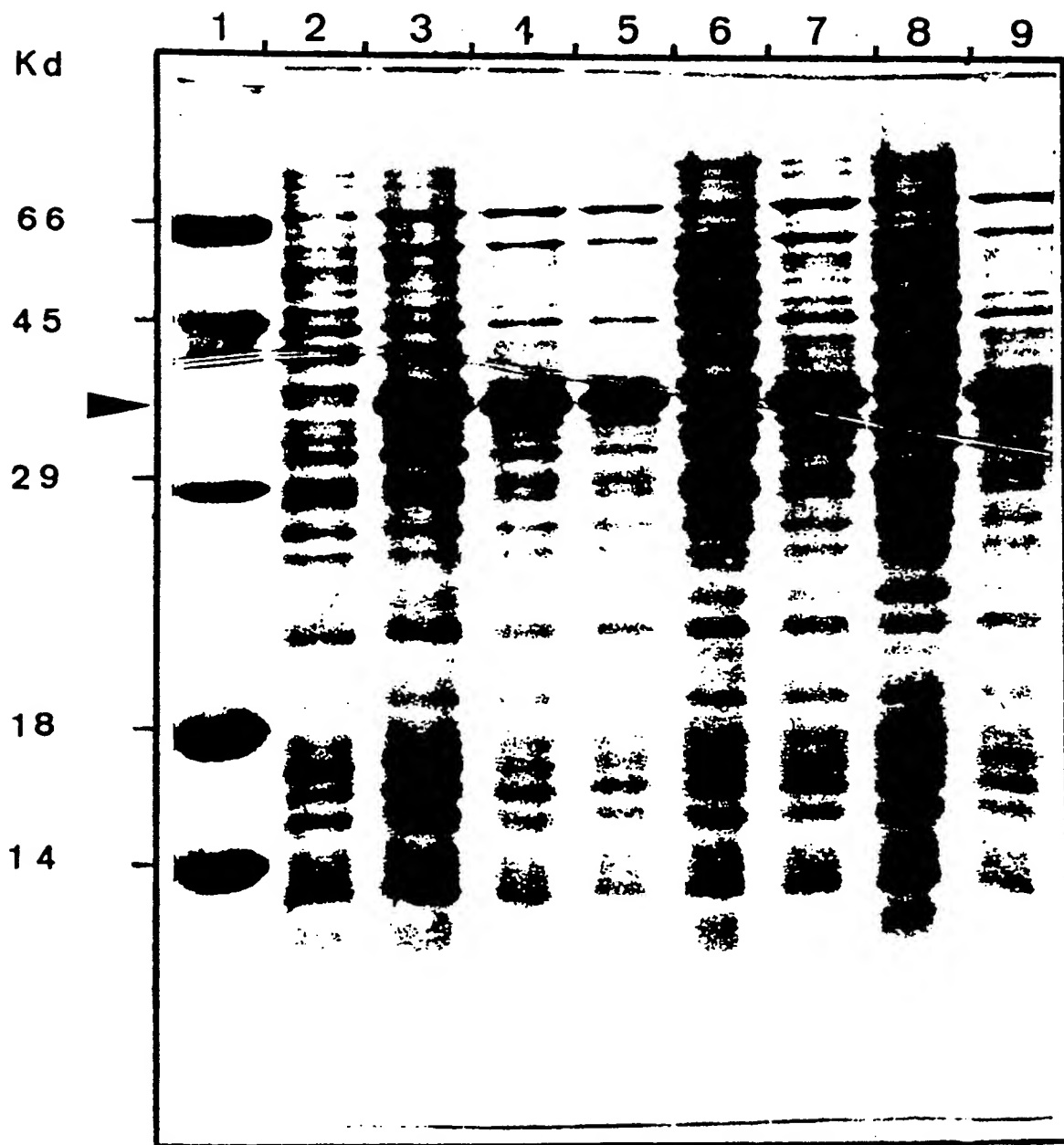


fig. 14a

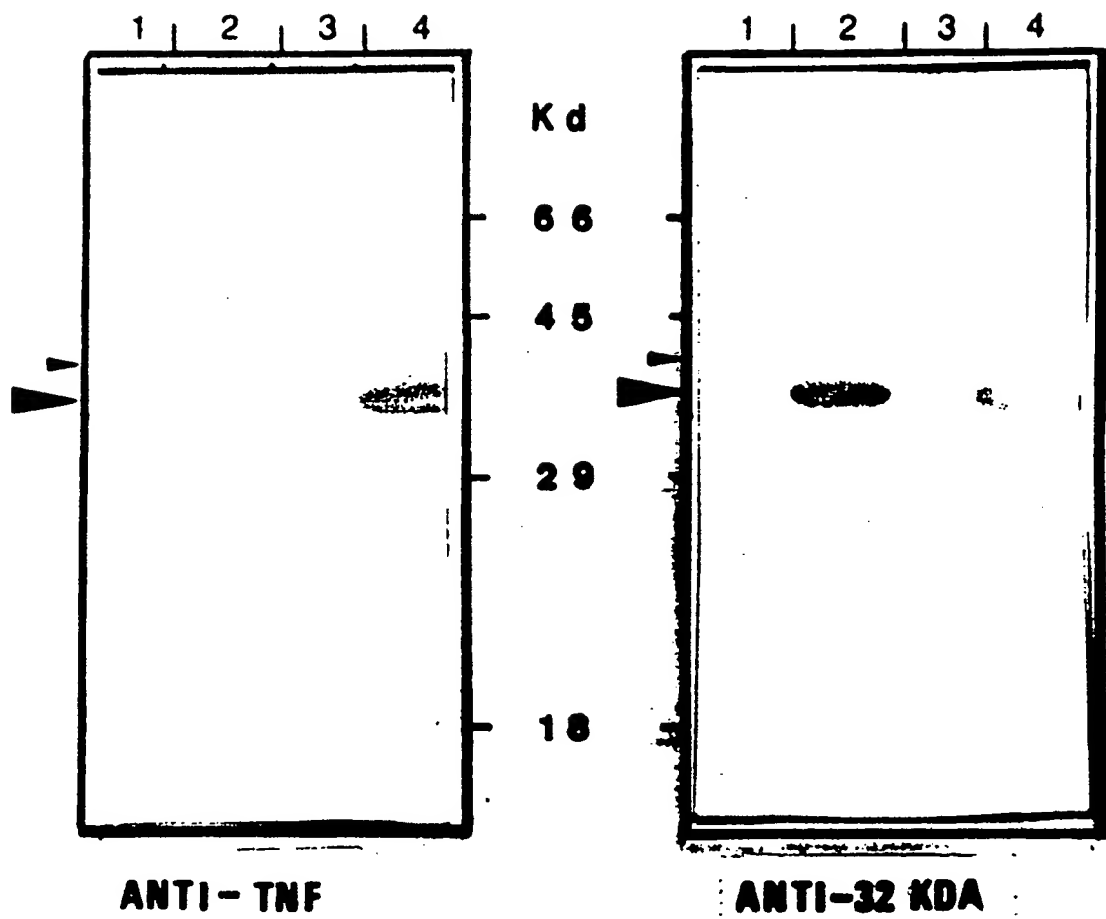


fig.14b

FIG. 15

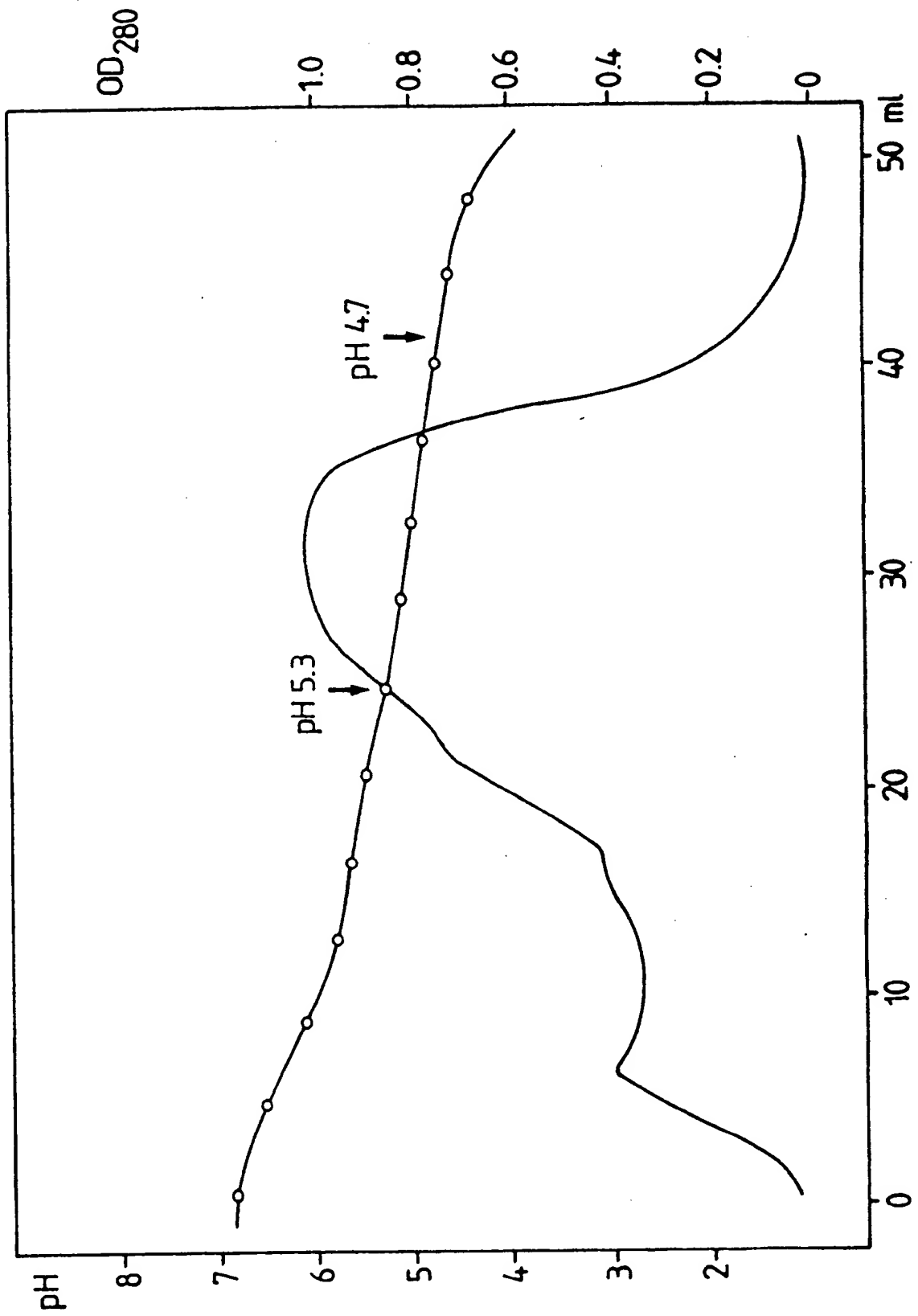


FIG. 16

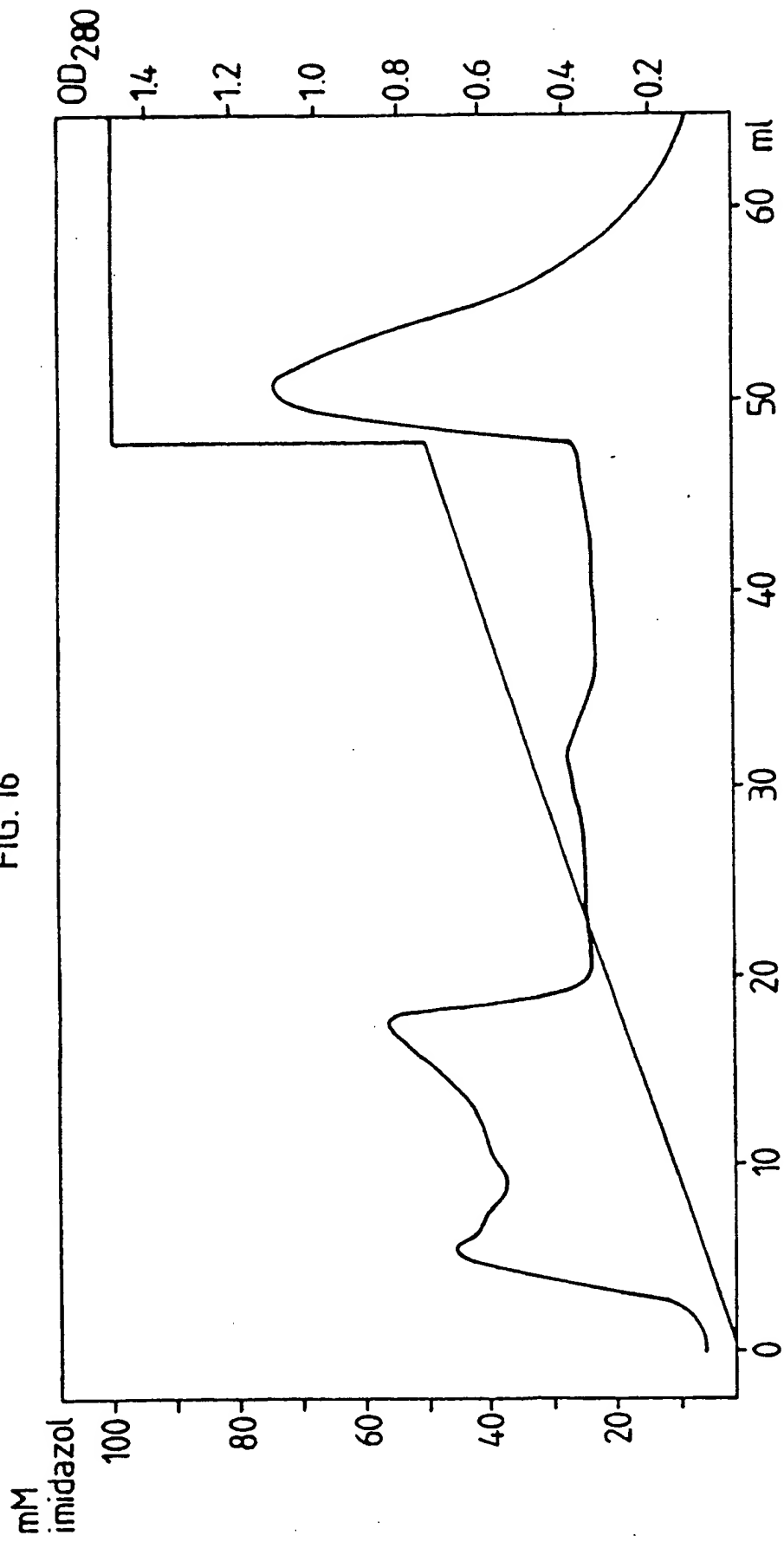
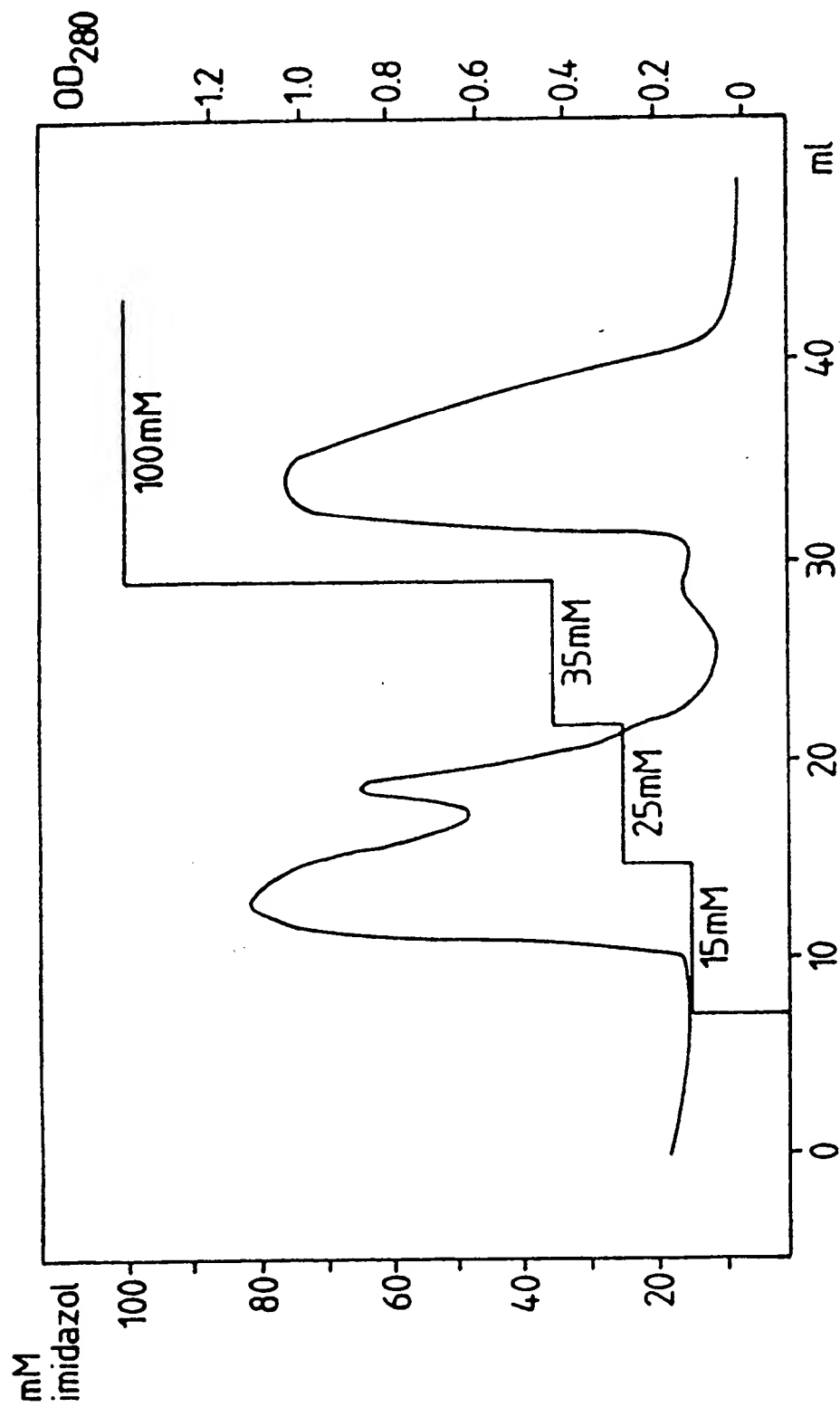


FIG. 17





EUROPEAN SEARCH REPORT

EP 90 40 2590

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,X	CHEMICAL ABSTRACTS, vol. 99, no. 11, 12th September 1983, page 413, abstract no. 86251m, Columbus, Ohio, US; H. TASAKA et al.: "Purification and antigenic specificity of alpha protein (Yoneda and Fukui) from Mycobacterium tuberculosis and Mycobacterium intracellulare", & HIROSHIMA J. MED. SCI. 1983, 32(1), 1-8 * Abstract * - - -	1-9,40,41	C 07 K 13/00 A 61 K 39/04 C 12 N 15/31 G 01 N 33.569 C 12 Q 1.68
D,X	JOURNAL OF CLINICAL MICROBIOLOGY, vol. 25, no. 7, July 1987, pages 1176-1180, American Society for Microbiology; M.L. COHEN et al.: "Expression of proteins of Mycobacterium tuberculosis in Escherichia coli and potential of recombinant genes and proteins for development of diagnostic reagents" * Whole document * - - -	10-22, 25-33, 35-39,43, 44	
X	BE-A-9 055 82 (INSTITUT PASTEUR DE BRABANT) * Pages 10,11; claims * - - -	35-39,45	
X	JOURNAL OF BACTERIOLOGY, vol. 170, no. 9, September 1988, pages 3847-3854, American Society for Microbiology; K. MATSUO et al.: "Cloning and expression of the Mycobacterium bovis BCG gene for extracellular alpha antigen" * Whole document * - - -	10-22,40, 41,43	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
Y	IDEM - - -	23,24,32, 34,42	C 07 K C 12 N A 61 K G 01 N C 12 Q
Y	EP-A-0 288 306 (McFADDEN) * Page 7, column 12, lines 2-18 * - - -	23,24,32, 34,42	
A	INT. ARCHS ALLERGY APPL. IMMUN., vol. 81, 1986, pages 307-314, S. Karger AG, Basel, CH; H.G. WIKER et al.: "MPB59, a widely cross-reacting protein of Mycobacterium bovis BCG" - - - - / -		
The present search report has been drawn up for all claims			
Place of search		Date of completion of search	Examiner
The Hague		20 December 90	SKELLY J.M.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone			
Y : particularly relevant if combined with another document of the same category			
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E : earlier patent document, but published on, or after the filing date			
D : document cited in the application			
L : document cited for other reasons			
& : member of the same patent family, corresponding document			



European
Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 40 2590

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	MICROBIAL PATHOGENESIS, vol. 2, 1987, pages 351-366, Academic Press Inc., London, GB; J. DE BRUYN et al.: "Purification, characterization and identification of a 32 kDa protein antigen of Mycobacterium bovis BCG"		

P,X	INFECTION AND IMMUNITY, vol. 57, no. 10, October 1989, pages 3123-3130, American Society for Microbiology; M. BORREMANS et al.: "Cloning, sequence determination, and expression of a 32-kilodalton-protein gene Mycobacterium tuberculosis"	1-45	

			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
The present search report has been drawn up for all claims			
Place of search		Date of completion of search	Examiner
The Hague		20 December 90	SKELLY J.M.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention</p> <p>E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons &: member of the same patent family, corresponding document</p>			

Abstract

The invention concerns a method for producing a species specific tuberculosis diagnostic from an extract or a centrifuged precipitate of a killed culture of *Mycobacterium* strains, advantageously *Mycobacterium bovis* or *Mycobacterium avium*, being purified from lipoids by lipid solvents, characterized by separating the individual protein-polysaccharide complexes, obtained from the concentrated filtrate of the culture or from an extract obtained by acidic hydrolysis of the bacterium precipitate, by electrophoresis or by adjusting the pH to correspond to the isoelectric point of the individual fractions, and eliminating the protein from the fraction comprising the polysaccharide portion providing the desired species specific tuberculin or serological reaction by cleaving the protein-polysaccharide bond, then separating the pure species specific polysaccharide by precipitation or gel filtration and converting it into a diagnostic reagent.